TESIS DOCTORAL

In situ analysis of the antibacterial activity of the essential oils on the oral biofilm

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HACE CONSTAR:

Como directora de la Tesis Doctoral que lleva por título "***In situ analysis of the antibacterial activity of the essential oils on the oral biofilm***", realizada por el licenciado en Odontología D. Víctor Quintas González, que cumple todos los requisitos para ser presentada y defendida ante el oportuno tribunal para optar al Grado de Doctor en Odontología por la Universidad de Santiago de Compostela.

Dra. Inmaculada Tomás Carmona *D. Víctor Quintas González*

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THESIS SUMMARY

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THESIS SUMMARY

"In situ analysis of the antibacterial activity of the essential oils on the oral biofilm"

The essential oils (EO) represent the oldest antiplaque and antigingivitis agent used clinically in dentistry. Studies performed *in situ* on the undisturbed dental plaque are considered of great value when assessing its efficacy on the oral biofilm. Models of 4-day of oral biofilm maturation have been found useful for the assessment of the antiplaque effect of oral antiseptics. Traditionally, the EO have been associated in a solution containing alcohol (T-EO) but recently, a new formulation without alcohol (Af-EO) has come to the market. Currently, there is little evidence on the *in situ* antibacterial activity of the Af-EO.

Firstly, the *in situ* antibacterial activity (immediate effect and substantivity) of a single mouthwash of T-EO was compared to the activity of the 0.2% chlorhexidine (0.2% CHX, positive control). This same comparison was done once again for the analysis of the antiplaque effect on a biofilm model of 4 days. Afterwards, these two comparisons were repeated, but this time assessing the effects of the Af-EO solutions *vs.* the T-EO solution.

An appliance was designed to hold six glass disks on the buccal sides of the lower teeth, allowing plaque-like biofilm (PL-biofilm) growth. Fifteen healthy volunteers wore the device for 48 hours, and then they performed a single mouthwash with T-EO (M-T-EO). Disks were removed 30 seconds and 1, 3, 5, and 7 hours later. The same procedure was repeated in the same subjects after application of a single mouthwash of sterile water (M-WATER, negative control) and a single mouthwash of 0.2% CHX (M-0.2% CHX, positive control). A 2-week washout period was established between the different rinsing protocols. After PL-biofilm vital staining (Live/Dead® BacLight™ Kit, Molecular Probes Inc. Leiden, The Netherlands), samples were analysed using a confocal laser scanning microscope (CLSM). Afterwards, this same protocol was repeated for the comparison between the Af-EO and the T-EO antiseptics.

The same designed appliance was used by another 15 healthy volunteers. Three different rinsing periods based on doing mouthwashes twice a day (1/0/1) during the 4-day period with T-EO (4D-T-EO), 0.2% CHX (4D-0.2% CHX; positive control) or sterile water (4D-WATER; negative

control) were applied. A 2-week washout period was established between the different rinsing protocols. At the end of each 4-day mouthwash period, samples were removed from the appliance. Posteriorly, after viability staining, samples were analysed using a CLSM, examining the thickness, covering grade (CG) and the bacterial viability (BV) of the PL-biofilm. Subsequently, this experiment was performed again for the comparative study between the Af-EO and the T-EO antiseptics.

In the experiment of a single antiseptic application, significant differences were not found over time after M-T-EO with regard to the basal thickness. At 30 seconds after M-T-EO, the mean level of BV was 1.18%, significantly lower than that detected in the basal sample (65.55%; $p<0.001$). After 7 hours, the antibacterial effect of M-T-EO was still patent with a 47.86% difference in BV compared to the basal sample ($p<0.001$). The M-T-EO obtained lower levels of BV at 1, 3 and 5 hours ($p<0.05$) but at 7 hours were as effective as 0.2% CHX (BV at 7 hours = 17.69 *vs.* 31.91%, $p>0.05$).

The 4D-T-EO and the 4D-0.2% CHX were significantly more effective than the 4D-WATER at reducing thickness, CG and BV of the PL-biofilm. No significant differences were found between the 4D-T-EO and 4D-0.2% CHX at reducing the BV (14.7% *vs*. 13.2%). However, the 4D-0.2% CHX showed more reduction than the 4D-T-EO in thickness (6.5 µm *vs.* 10.0 µm; $p<0.05$) and CG by the PL-biofilm (20.0% *vs.* 54.3%; $p<0.001$).

The thickness of the PL-biofilm after rinsing was not affected by any of the EO formulations and ranged from 18 to 24 µm. The T-EO and Af-EO solutions had a similar antibacterial effect, reducing BV after a single mouthwash compared to the WATER, and keeping it below baseline levels up to 7 hours post-rinse ($p<0.001$) After 4 days, both antiseptics were significantly more effective than the WATER, reducing the thickness, CG and BV of the PL-biofilm. Both EO solutions presented similar antibacterial activity (BV = 26.27% *vs.* 31.08%, $p>0.016$). The Af-EO rinse, however, led to more significant reductions in the thickness (9.92 µm *vs.* 7.90 µm, $p = 0.012$) and CG of the PL-biofilm (46.61% *vs.* 33.36%, $p<0.016$).

A single mouthwash of the traditional formulation of EO presented very high immediate antibacterial effect *in situ* and a substantivity which lasted for at least 7 hours after its application over a 2-day PL-biofilm. These results were even better than those observed with 0.2% CHX under the same conditions.

The T-EO and 0.2% CHX showed a very high antiplaque effect. Although the 0.2% CHX showed better results with regard to reducing the thickness and covering grade by the biofilm, both antiseptics showed a high and similar antibacterial activity.

Both EO antiseptics had very high immediate antibacterial activity and substantivity *in situ* on the 2-day PL-biofilm after a single mouthwash. In the 4-day PL-biofilm, both EO formulations demonstrated a very high antiplaque effect *in situ*, although the alcohol-free formula performed better at reducing the biofilm thickness and CG.

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RESUMEN DE LA TESIS

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RESUMEN DE LA TESIS

"Análisis in situ de la actividad antibacteriana de los aceites esenciales sobre el biofilm oral"

INTRODUCCIÓN

Las bacterias son la forma predominante de vida en la Tierra ya que son ubicuas, muy heterogéneas y se adaptan rápidamente a los cambios en el ambiente que las circunda. Por todas estas razones las bacterias están caracterizadas como organismos vivos prácticamente inextinguibles. En la naturaleza, se estima que más del 95% de las bacterias viven en asociación formando biopelículas (biofilms) [1].

Las bacterias en la naturaleza existen principalmente como miembros de comunidades de biofilms y no como células aisladas [1]. Los biofilms se han descrito como auténticas "Ciudades de Microbios" donde las bacterias viven en comunidad y se relacionan entre ellas. Esta compleja asociación hace que las bacterias sean de 10 a 1000 veces más resistentes frente a agentes microbianos que las células bacterianas en fase planctónica [2].

La placa dental es un tipo especializado de biofilm, siendo uno de los que más se han estudiado. Aparte de bacterias, esta biopelícula contiene una matriz de polímeros de origen bacteriano y salival [2].

Las bacterias siempre están presentes en el ecosistema oral, tanto en situaciones de salud, como de enfermedad. El estudio de este biofilm es de gran importancia ya que algunas especies contribuyen a mantener la salud y otras son causa potencial de enfermedad. El biofilm oral está implicado en la mayoría de las enfermedades y procesos que tienen lugar en la cavidad oral, tales como caries, halitosis, enfermedad periodontal y también en procesos de cicatrización después de manipulaciones odontológicas [3].

Los modelos para el estudio de biofilm *in vitro* han sido muy importantes en la historia del biofilm oral. Sin embargo, la comunidad científica ha reconocido sus limitaciones, recomendando la interpretación cautelosa de sus resultados [4-6]. Se establece así la necesidad de desarrollar modelos de biofilm de formación *in situ*, que a su vez, permitan su análisis sin distorsión de su estructura [4, 6-8].

De este modo, surgen en la literatura científica diversos dispositivos intraorales para conseguir la formación de un biofilm similar a la placa dental que se ha denominado “biofilm de placa dental” (“plaque-like biofilm, PL-biofilm”). En estos dispositivos se incorporan diversos sustratos como base para la formación del PL-biofilm, tales como: el esmalte humano, esmalte bovino, hidroxiapatita, vidrio pulido, dentina bovina, raíces de dientes humanos o incluso materiales de restauración e implantes.

Las técnicas de microscopía basadas en la fluorescencia permiten analizar el biofilm sin someterlo a agresivos protocolos que modifican su estructura y características. Entre los microscopios de fluorescencia destaca el microscopio de barrido láser confocal (confocal laser scanning microscope, CLSM), el cual permite obtener imágenes de la muestra realizando un barrido plano a plano a lo largo de toda la profundidad del espécimen. Esto permite obtener una serie de secciones del grosor del espécimen. En la literatura se han utilizado múltiples tinciones para el estudio del biofilm oral, desde la hibridación *in situ,* que utiliza sondas específicas para ADN, hasta otras que con la combinación de fluorocromos, permiten estudiar la viabilidad bacteriana (bacterial viability, BV) presente en la muestra. Entre los más utilizados destacan el di-acetato de fluoresceína (fluorescein di-acetate, FDA) combinado con bromuro de etidio (etidium bromide, EtBr) y el SYTO 9 combinado con ioduro de propidio (propidium iodide, PI). Este último, en el formato Live/Dead® BacLight™ Kit de BV, (Molecular Probes Inc, Leiden, The Netherlands) ha sido utilizado por nuestro grupo de investigación en múltiples ocasiones [9-17], así como por otros expertos [18-25] consiguiendo unos resultados coherentes y demostrando su idoneidad para el estudio del biofilm oral.

El acúmulo de placa dental sobre la superficie de los dientes es una de las causas principales de aparición de las dos enfermedades infecciosas más prevalentes en el ser humano -la caries y la enfermedad periodontal-. Aunque la prevención y el control de estas enfermedades puede ser logrado mediante la eliminación mecánica diaria de estos biofilms, muchos individuos son incapaces o no practican estas medidas de una manera regular y eficiente. Es por ello, que los métodos químicos son vistos como una opción muy interesante para complementar y mejorar así las medidas mecánicas y los procedimientos terapéuticos [26-28].

El control del biofilm oral es un punto clave en la clínica dental en todas las ramas de la odontología, tanto en la periodoncia, cirugía, operatoria e, incluso, ortodoncia. Dentro del control químico de la placa dental destacan los antisépticos de uso oral, que son considerados un importante complemento a las medidas de higiene mecánicas como el cepillado dental. Entre los más utilizados clínicamente destacan la clorhexidina (chlorhexidine, CHX) [29] y los aceites esenciales (essential oils, EO) [30].

Las soluciones de EO están caracterizadas por tener en su formulación una concentración fija de cuatro elementos como son: el eucaliptol, el timol, el mentol y el metil-salicilato [31]. Estos compuestos tienen un complejo mecanismo de acción antibacteriana [32], aunque ejercen su principal efecto a nivel de la membrana y las paredes celulares bacterianas, éste induce una reacción en cadena de otros eventos [33]. Los EO tradicionalmente se han venido comercializando en una solución hidroalcohólica (traditional essential oils, T-EO) que, si bien no es suficiente para ejercer una acción antibacteriana por sí misma, sí lo es para disolver los EO [34]. Hace pocos años surgió una nueva formulación de EO (alcohol-free essential oils, Af-EO) que, aunque contenía los cuatro elementos antes mencionados, utilizaba para la estabilización de la fórmula un componente no alcohólico como es el lauril sulfato de sodio. Esta nueva formulación fue considerada una posible alternativa a la solución tradicional, sobre todo en pacientes pediátricos o para aquellos pacientes con especial riesgo de desarrollar cáncer orofaríngeo [35, 36]. Sin embargo, la ausencia de literatura sobre su eficacia sobre la placa dental limita su utilización bajo criterios de evidencia científica.

El otro compuesto más utilizado en odontología es la CHX, cuya actividad antibacteriana y antiplaca ha sido ampliamente estudiada y constatada [29]. Como los EO, la CHX centra su principal efecto antibacteriano en la alteración de la membrana celular bacteriana [37].

JUSTIFICACIÓN Y OBJETIVOS

Los EO y la CHX son, probablemente los antisépticos más estudiados y populares de todos los comercialmente disponibles. Existen muchos estudios que analizan la actividad antibacteriana y los efectos antiplaca de estos antisépticos sobre el biofilm oral. Sin embargo, la mayor parte de estas investigaciones tiene un carácter eminentemente clínico, ya que el análisis de efectividad de los antisépticos es realizado aplicando índices clínicos de placa, obteniendo unos resultados muy heterogéneos.

Otros autores estudiaron el biofilm oral a nivel microbiológico; midiendo las unidades formadoras de colonias, describiendo el biofilm oral cualitativamente mediante el microscopio electrónico de barrido (scanning electron microscope, SEM) o el de transmisión (transmission electron microscope, TEM). La mayor desventaja de estas técnicas es la imposibilidad de analizar las características del biofilm oral de forma precisa debido a la necesidad de romper su delicada arquitectura tridimensional.

El CLSM en conjunción con técnicas de tinción de fluorescencia dual (análisis de las bacterias vivas/muertas) han demostrado su eficacia y fiabilidad en el estudio del biofilm oral no desestructurado *in situ.* Además, los dispositivos ideados para el desarrollo del biofilm oral *in situ,* han mejorado su diseño y confort, interfiriendo mínimamente en la vida de los voluntarios [38].

Existen pocos estudios en la literatura en los que los efectos antibacterianos de los EO sobre el biofilm oral *in situ* no desestructurado hayan sido evaluados mediante el CLSM junto con técnicas de BV [23, 24]. Además, la aparición de formulaciones de EO sin alcohol (Af-EO) hace indispensable la evaluación de su actividad antibacteriana con respecto a la formulación tradicional sin alcohol (T-EO).

Por todas estas razones, los objetivos de la presente Tesis son:

1. Evaluar el efecto antibacteriano *in situ* y la sustantividad de un único enjuague con los T-EO sobre el biofilm oral de 2 días no desestructurado formado *de novo* hasta 7 horas después de su aplicación, usando CLSM y una solución de fluorescencia dual.

2. Evaluar el efecto antiplaca *in situ* de enjuagues diarios de los T-EO a corto plazo sobre el biofilm oral de 4 días no desestructurado formado *de novo*, usando CLSM y una solución de fluorescencia dual.

3. Comparar el efecto antibacteriano *in situ* (efecto inmediato, sustantividad y efecto antiplaca) de los T-EO y de la nueva formulación sin alcohol –Af-EO- sobre el biofilm oral de 2 y 4 días no desestructurado formado *de novo*, usando CLSM y una solución de fluorescencia dual.

OBJETIVO 1: EFECTO ANTIBACTERIANO INMEDIATO Y SUSTANTIVIDAD DE UN ÚNICO ENJUAGUE DE ACEITES ESENCIALES EN UN MODELO *IN SITU* DE BIOFILM ORAL NO DESESTRUCTURADO: UN ENSAYO CLÍNICO RANDOMIZADO

MATERIAL Y MÉTODOS:

Se planteó realizar un estudio clínico a doble ciego, aleatorizado en el que 15 voluntarios adultos sanos cumplieron los siguientes criterios de inclusión: edad entre 20 y 45 años, con un mínimo de 24 dientes permanentes, sin caries activas, sin signos de gingivitis o periodontitis, sin prótesis ni dispositivos ortodóncicos, no fumadores, que no hubieran tomado antibióticos al menos durante los 3 meses previos al inicio del estudio, que no usaran antisépticos de forma rutinaria, que no presentaran enfermedades sistémicas que afectaran la formación o composición de la saliva. Todos ellos portaron en la cavidad oral durante 2 días un dispositivo de férulas superpuestas fabricado de forma individualizada y con seis discos cada uno. Después de este período, los voluntarios realizaron de forma supervisada un único enjuague con T-EO (M-T-EO), CHX al 0,2% (M-0,2% CHX, control positivo) o con agua estéril (M-WATER, control negativo). Todos los voluntarios efectuaron los tres enjuagues en tres momentos distintos entre los cuales se dejó un período de lavado de al menos 2 semanas.

El día del análisis, los discos se retiraron secuencialmente del dispositivo intraoral antes del enjuague (toma basal), a los 30 segundos (inmediata), a la hora, 3 horas, 5 horas y 7 horas post-enjuague. Las

muestras se analizaron con el CLSM, después de la tinción con SYTO 9 y PI (Live/Dead® BacLight™ Kit, Molecular Probes Inc, Leiden, The Netherlands). Se evaluó el grosor y la BV del biofilm oral.

Para el análisis estadístico de los datos de grosor y BV, se verificó la distribución normal mediante el test de Kolmogorov-Smirnov. Se utilizó el test ANOVA de un factor para las comparaciones intra-enjuague para todas las muestras de biofilm. El test ANOVA de dos factores se aplicó para las comparaciones inter-enjuague (distinguiendo entre las tres capas de biofilm). Se utilizaron las comparaciones por pares (con el ajuste de Bonferroni) para el análisis de los resultados intra- e inter-enjuague. Se estableció como significativo un valor de *p* inferior a 0,05.

RESULTADOS:

La media de grosor del PL-biofilm en la situación basal fue de 22,15 µm. No se detectaron cambios significativos en el grosor del biofilm después del M-T-EO con respecto a su basal. Sin embargo, después del M- 0.2% CHX sí que se obtuvieron niveles inferiores de grosor con respecto a su basal y a las mediciones después del M-T-EO.

La BV basal del PL-biofilm fue de 73,59%. Tanto los T-EO como la 0,2% CHX se mostraron eficaces a la hora de reducir los niveles de BV frente a su basal desde la toma inmediata a las 7 horas post-enjuague.

En comparación con los valores obtenidos 30 segundos después de los M-0,2% CHX y M-T-EO, se produjo una recuperación significativa de la viabilidad de la población bacteriana en las muestras posteriores (después de las 3 y 5 horas post-enjuague, respectivamente). Comparando los efectos de la 0,2% CHX con los de los T-EO, estos últimos presentaron porcentajes de BV menores hasta las 7 horas después de la aplicación, obteniendo diferencias significativas desde la primera hora hasta 5 horas después del enjuague.

Diferenciando entre las tres capas de biofilm, los T-EO obtuvieron una BV significativamente inferior a la 0,2% CHX en las capas medias y profundas del biofilm desde la muestra de 1 hora después del enjuague hasta las 7 horas.

DISCUSIÓN:

Como muestran los resultados de la serie actual, una sola aplicación de EO no fue eficaz para reducir el grosor del PL-biofilm. Estos hallazgos coinciden con los previamente descritos por Dong et al. en un biofilm de 48 horas [23], en el cual no encontraron diferencias significativas en el grosor de la biopelícula con respecto a la muestra basal después de aplicar un M-T-EO. Estos hallazgos son también coherentes con un estudio *in vitro* realizado por Sliepen et al. [39], quienes observaron que los T-EO no causaron casi ningún cambio en la estructura del biofilm, el grosor y la cobertura superficial. En cuanto a la CHX al 0,2%, se encontraron diferencias estadísticamente significativas en el grosor del PL-biofilm en comparación con los T-EO a los 30 segundos, 1, 3 y 7 horas después del enjuague bucal, lo que podría sugerir un cierto efecto antiplaca de la CHX al 0,2% después de una sola aplicación antiséptica.

Con una viabilidad basal similar entre los tres enjuagues bucales (media de 73,6%), se detectó un elevado efecto antibacteriano inmediato después de la aplicación de los T-EO. Esta actividad inmediata fue muy alta en comparación con la obtenida en una investigación similar publicada por Gosau et al. [24], ya que en este estudio, la BV después del uso de los T-EO fue de alrededor del 20%, mientras que en la presente serie, la viabilidad fue del 1% a los 30 segundos después del M-T-EO.

En la presente serie, la actividad antimicrobiana de los T-EO fue detectable hasta 7 horas después de la aplicación del enjuague bucal, cuando la reducción en la BV era todavía del 61%. En este estudio, también se apreció que una única aplicación de T-EO era eficaz para mantener bajos niveles de BV en el PL-biofilm. No hubo diferencias estadísticamente significativas en la BV hasta 5 horas en comparación con a los 30 segundos post-enjuague, lo que indica una potente y persistente actividad antibacteriana hasta ese momento. Hasta ahora, se han realizado dos estudios sobre la sustantividad de los T-EO en el biofilm oral. El primero encontró una BV del 21,3% a los 30 minutos después del enjuague bucal [40], mientras que el otro, realizado por Fine [41], mostró una reducción de la BV del 88% después de 12 horas después de la aplicación. Ambos estudios utilizaron un modelo *in situ* de placa dental desestructurada, por lo que estos resultados no son totalmente comparables. En cuanto al

control positivo (0,2% CHX), hubo diferencias significativas desde 1 hora hasta las 5 horas después del enjuague, siendo los T-EO más eficaces en el mantenimiento de valores bajos de BV. Estos hallazgos no están descritos en la literatura disponible debido a la falta de estudios que evalúen la sustantividad de los T-EO sobre el biofilm oral en comparación con la CHX.

Otro aspecto interesante de este estudio es la mayor capacidad de penetración en el PL-biofilm de los T-EO en contraste con la CHX al 0,2%. Se detectaron diferencias estadísticamente significativas en la reducción de la BV en la capa 3 (la más profunda) entre ambos antisépticos, a partir de las muestras inmediatas y de 1 hora, pero más aún, a las 7 horas después del único enjuague. Este hallazgo indica que la capacidad de penetración de una sola aplicación de T-EO es mayor que la que muestra una sola aplicación de CHX al 0,2% [16]. Como Pan et al. [40] previamente describió, estos resultados confirman la capacidad del enjuague bucal de T-EO para penetrar en la placa y ejercer su actividad bactericida *in situ* rápidamente. Aparte de eso, los T-EO mantienen su actividad antimicrobiana en las capas más profundas (más cercanas a la superficie teórica del diente) durante más tiempo.

OBJETIVO 2: EFECTO ANTIPLACA DE UN PROTOCOLO DE ENJUAGUE DE 4 DÍAS SOBRE UN MODELO *IN SITU* DE BIOFILM NO DESESTRUCTURADO: UN ENSAYO CLÍNICO RANDOMIZADO.

MATERIAL Y MÉTODOS:

Se planteó realizar un estudio clínico a doble ciego, aleatorizado en el que 15 voluntarios adultos sanos cumplieron los siguientes criterios de inclusión: edad entre 20 y 45 años, con un mínimo de 24 dientes permanentes, sin caries activas, sin signos de gingivitis o periodontitis, sin prótesis ni dispositivos ortodóncicos, no fumadores, que no hubieran tomado antibióticos al menos durante los 3 meses previos al inicio del estudio, que no usaran antisépticos de forma rutinaria, que no presentaran enfermedades sistémicas que afectaran a la formación o composición de la saliva. Todos ellos portaron en la cavidad oral durante 4 días un dispositivo de férulas superpuestas fabricado de forma individualizada y con seis discos cada uno. Durante el período de 4 días, los voluntarios

realizaron de forma no supervisada dos enjuagues diarios (1/0/1) con: T-EO (4D-T-EO), CHX al 0,2% (4D-0,2% CHX) o agua estéril (4D-WATER). Todos los voluntarios utilizaron los tres enjuagues en tres momentos distintos entre los cuales se dejó un período de lavado de al menos 2 semanas.

El día del análisis, los discos se retiraron secuencialmente del dispositivo intraoral siendo analizados uno tras otro mediante el CLSM, después de la tinción con SYTO 9 y PI (Live/Dead® BacLight™ Kit, Molecular Probes Inc, Leiden, The Netherlands). Se evaluó el grosor, el área ocupada (covering grade, CG) y la BV del biofilm oral.

Para el análisis estadístico de los datos de grosor, BV y área ocupada, se verificó la distribución normal mediante el test de Kolmogorov-Smirnov. Se utilizó el test ANOVA de un factor para las comparaciones intra-enjuague para todas las muestras de biofilm. El test ANOVA de dos factores se aplicó para las comparaciones inter-enjuague (distinguiendo entre las tres capas de biofilm). Se utilizaron las comparaciones por pares (con el ajuste de Bonferroni) para el análisis de los resultados intra- e inter-enjuague. Se estableció como significativo un valor de p inferior a 0,05.

RESULTADOS:

Los protocolos de 4D-T-EO y 4D-0,2% CHX resultaron ser significativamente efectivos con respecto al 4D-WATER a la hora de reducir el espesor del biofilm (9,99 ± 3,27 µm y 6,48 ± 1,82 µm, respectivamente *vs.* 23,44 ± 4,78 µm; $p<0,001$ para todas las comparaciones) El CG también se redujo significativamente con el 4D-T-EO y 4D-0,2% CHX con respecto al 4D-WATER (54,32 ± 17,49% y 20,01 ± 16,52%, respectivamente *vs.* 75,17 ± 16,51%; $p<0,05$ y $p<0,001$, respectivamente).

Cuando se compararon ambas soluciones antisépticas, la CHX al 0,2% presentó una mayor actividad antibacteriana que los T-EO reduciendo el grosor del biofilm ($p<0,05$) y el CG ($p<0,001$) después de un período de 4 días de enjuagues.

Ambos protocolos de 4D-T-EO y 4D-0,2% CHX resultaron ser significativamente efectivos con respecto al 4D-WATER disminuyendo la BV (14,67 ± 5,54% y 13,19 ± 18,09%, respectivamente *vs.* 56,53 ± 14,40%; $p<0,001$).

Cuando se compararon ambas soluciones antisépticas, los EO fueron tan eficaces como la CHX al 0,2% en lo que respecta a la reducción de la BV después de un período de 4 días de enjuagues.

Respecto a la BV del biofilm por capas, después de los protocolos con el control negativo y los 4D-T-EO, la viabilidad fue significativamente mayor (p<0,001) en las capas superficiales que en las profundas. Por otro lado, tras el período con 4D-0,2% CHX, no hubo diferencias de BV entre las tres capas del biofilm.

En cuanto a las comparaciones de las mismas capas de biofilm entre los diferentes protocolos de enjuague, ambas soluciones antisépticas, T-EO y CHX al 0,2%, presentaron menos BV en todos ellas en relación con el período 4D-WATER, siendo las diferencias más marcadas entre las capas superficiales (p<0,001) y menos pronunciadas entre las capas profundas (p<0,05). No hubo diferencias en términos de BV entre las mismas capas después de los regímenes de 4D-T-EO y 4D-0,2% CHX, siendo superiores los valores en las capas superficiales en ambos casos.

DISCUSIÓN:

El grosor del PL-biofilm *in situ* después de 4 días de aplicación de CHX al 0,2% y T-EO fue de 6,48 µm y 9,99 µm, respectivamente. Existen varios estudios [42-46] en los que se analizó el grosor del PL-biofilm. Los resultados difieren entre ellos dependiendo de la técnica utilizada para su medición, tipo y concentración de los enjuagues bucales y su duración.

El único estudio, aparte de la presente serie, que compara la eficacia antibacteriana de T-EO y CHX es el de Jentsch et al. [46]. Estos autores midieron el grosor del PL-biofilm desarrollado sobre la superficie de cortes de esmalte después de un período de enjuague de 4 días. A diferencia de los hallazgos reportados en el presente estudio, no obtuvieron diferencias en el efecto de reducir el grosor de PL-biofilm entre ambas soluciones antisépticas, T-EO y CHX (15,13 µm y 16,67 µm después de 4 días). Nuestros resultados son similares a los obtenidos por otros autores después de la aplicación de 0,2% CHX (8,6 µm después de 2 días [45] y 11,91 µm después de 5 días [43]).

El CG por el PL-biofilm en combinación con su grosor está directamente relacionado con la capacidad antiplaca de un agente antiséptico. Según el conocimiento del autor, no existen artículos publicados que analicen estos parámetros en un PL-biofilm de 4 días *in situ*. Por otro lado, existen algunos estudios *in vitro* sobre este tema y uno de ellos obtuvo resultados similares a los de la presente serie. Es uno de Al-Ahmad et al. [47], quienes obtuvieron porcentajes de CG de 77% y 7% con control negativo y CHX al 0,2% después de 4 días de uso, respectivamente (en la presente serie, 75% y 20%, respectivamente).

En cuanto a la BV después de los protocolos de enjuague bucal con T-EO y CHX al 0,2%, las reducciones de BV son similares a los que se han reportado anteriormente [43, 45]. En la literatura, para la CHX al 0,2% y el fluoruro de estaño, estos valores de viabilidad fueron del 62% y 64%, respectivamente [43, 45]. En la presente serie, la reducción de BV fue similar entre ambos antisépticos (74% para T-EO y 77% para CHX al 0,2%). Una vez más, la BV más baja se encontró en las capas más profundas del PL-biofilm.

Hay dos estudios *in situ* de 4 días que compararon el efecto de los T-EO y la CHX en términos de recuentos bacterianos [48, 49]. En estos estudios, se realizó el control de la placa, y se determinaron las especies cultivables usando técnicas de cultivo en placa. Finalmente, concluyeron que los T-EO y la CHX al 0,12% y 0,1% (las concentraciones usadas, respectivamente) tenían efectos antiplaca similares. En la presente serie, aunque el T-EO y la CHX al 0,2% mostraron una actividad antibacteriana muy alta y similar, esta última fue más potente al inhibir la formación de PL-biofilm, ya que tanto el grosor como el CG fueron considerablemente inferiores en su caso.

OBJETIVO 3: ACTIVIDAD ANTIBACTERIANA DE LOS ACEITES ESENCIALES CON Y SIN ALCOHOL SOBRE UN MODELO *IN SITU* DE BIOFILM NO DESESTRUCTURADO: UN ENSAYO CLÍNICO RANDOMIZADO

MATERIAL Y MÉTODOS:

Se planteó realizar un estudio clínico a doble ciego, aleatorizado en el que 18 voluntarios adultos sanos cumplieron los siguientes criterios de inclusión: edad entre 20 y 45 años, con un mínimo de 24 dientes permanentes, sin caries activas, sin signos de gingivitis o periodontitis, sin prótesis ni dispositivos ortodóncicos, no fumadores, que no hubieran tomado antibióticos al menos durante los 3 meses previos al inicio del estudio, que no usaran antisépticos de forma rutinaria, que no presentaran enfermedades sistémicas que afectaran la formación o composición de la saliva. Se confeccionó para todos ellos un dispositivo de férulas superpuestas fabricado de forma individualizada y con seis discos cada uno. El estudio estuvo dividido en dos tests: el primero, en el que se evaluó el efecto inmediato y la sustantividad de una única aplicación de dos formulaciones de EO con alcohol (T-EO) y sin alcohol (Af-EO) en un modelo de PL-biofilm *in situ* de 2 días de evolución; el segundo, en el que se evaluó el efecto antiplaca de aplicaciones diarias de ambas formulaciones de EO en un modelo de PL-biofilm *in situ* de 4 días. Se siguieron los mismos protocolos de aplicación de los enjuagues de los Objetivos 1 y 2, respectivamente.

En el test 1, el día del análisis los discos se retiraron secuencialmente del dispositivo intraoral antes del enjuague (toma basal), a los 30 segundos (inmediata), a la hora, 3 horas, 5 horas y 7 horas. Las muestras se analizaron con el CLSM, después de la tinción con SYTO 9 y PI (Live/Dead® BacLight™ Kit, Molecular Probes Inc, Leiden, The Netherlands). Se evaluó el grosor y la BV del biofilm oral.

En el Test 2, el día del análisis, los discos se retiraron secuencialmente del dispositivo intraoral siendo analizados uno tras otro mediante el CLSM, después de la tinción con SYTO 9 y PI. Se evaluó el grosor, el CG y la BV del biofilm oral.

Los análisis estadísticos se realizaron utilizando el software R [42]. La prueba de Shapiro-Wilk se realizó para analizar la normalidad de las variables cuantitativas asociadas con el PL-biofilm (grosor, CG y BV), mostrando en la mayoría de estos parámetros microscópicos una distribución no normal en ambos tests.

En el Test 1 (sustantividad) y en el Test 2 (efecto antiplaca), se utilizó la prueba de Friedman para las comparaciones intra-enjuague e inter-enjuague utilizando todas las muestras de PL-biofilm (incluyendo la diferenciación entre las tres capas de biofilm). En ambos tests se utilizó la prueba de Wilconxon para las comparaciones por pares (con el ajuste de Bonferroni) de los resultados intra- e inter-enjuague (incluyendo la diferenciación entre las tres capas de biofilm). El nivel de significación establecido fue un valor de *p* inferior a 0,05. En el Test 1, los valores de *p* corregidos por Bonferroni fueron <0,003 y <0,016, y en el Test 2, este valor fue <0,016.

RESULTADOS:

Ninguno de los dos antisépticos a base de EO tuvo la capacidad de reducir el grosor del PL-biofilm de 48 horas después de una sola aplicación. Sus grosores basales fueron: 21,81 ± 5,28 µm y 20,71 ± 4,13 µm, respectivamente. Después de un solo enjuague, los grosores se redujeron ligeramente (20,19 ± 3,62 µm y 18,58 ± 3,14 µm, respectivamente), pero no alcanzaron significación estadística.

Ambas formulaciones de EO lograron resultados similares de BV en todos los puntos de tiempo medidos. De hecho, no se encontraron diferencias entre ellos desde la muestra inmediata (30 segundos) hasta las 7 horas post-enjuague. Las formulaciones de EO fueron eficaces para reducir la BV después de un único enjuague con respecto a la situación basal (BV a los 30 segundos para M-T-EO y M-Af-EO = 6,53 ± 7,60% y 4,13 ± 3,89%; *p*<0,001 para ambas comparaciones). Estos porcentajes fueron significativamente inferiores que los de M-WATER (62,39 ± 8,17%; p<0,001). Ambas soluciones fueron capaces de mantener la BV por debajo de los niveles basales durante 7 horas (BV a las 7 horas para M-T-EO y M-Af-EO = 18,20 ± 9,38% y 20,10 ± 10,27%, respectivamente; *p*<0,001). De nuevo, estos hallazgos fueron más bajos de forma significativa que los de M-WATER (BV a las 7 horas = 76,78 ± 4,40%; *p*<0,001).

La recuperación de la BV con respecto a la muestra de 30 segundos, no se logró hasta 7 horas después del uso de M-T-EO (BV a 30 segundos y 7 horas post-enjuague= 6,53 ± 7,60% y 18,20 ± 9,38; *p* <0,001). Sin embargo, para la solución M-Af-EO, se identificó una recuperación significativa de la viabilidad ya en la muestra de las 3 horas (BV a 30 segundos y 3 horas post-enjuague= 4,13 ± 3,89% y 12,35 ± 8,86%; *p*<0,001).

Cuando se trata de diferenciar entre las tres capas de biofilm, los dos antisépticos de EO presentaron niveles de BV más bajos en todas las capas. No se encontraron diferencias significativas en la BV para la misma capa de biofilm entre las formulaciones de EO, siendo las capas superficiales generalmente más viables que las profundas en todas las muestras. No hubo diferencias significativas entre las tres capas para el M-T-EO (BV de las capas 1, 2 y 3 a los 30 segundos = 6,67 ± 6,80% *vs.* 5,63 ± 8,15% *vs.* 7,29 ± 9,17%, respectivamente; *p*>0,016) o entre las capas más profundas para el enjuague M-Af-EO (BV de las capas 2 y 3 a los 30 segundos = 3,22 ± 3,24% *vs.* 3,51 ± 4,94%, respectivamente; *p*>0,016)

Los enjuagues de Af-EO fueron más efectivos que la formulación de T-EO reduciendo el grosor de la biopelícula oral después de 4 días de uso (grosor tras 4D-T-EO frente a 4D-Af-EO = 9,92 ± 2,87 µm frente a 7,9 ± 2,91 µm; *p* = 0,012), pero ambas soluciones fueron más potentes que el control negativo (grosor tras 4D-WATER = 22,76 ± 6,21 µm; *p*<0,001).

El enjuague de Af-EO fue más eficaz que la solución de T-EO reduciendo el CG del biofilm oral después de 4 días de uso (CG para 4D-T-EO frente a 4D-Af-EO = 46,61 ± 19,12% frente a 33,36 ± 12,01%; *p* = 0,001), pero ambas soluciones fueron significativamente más eficaces que el control negativo (CG para 4D-WATER = 73,92 ± 17,49%; *p*<0,001)

Las formulaciones de T-EO y Af-EO después de 4 días de uso fueron eficaces para mantener la BV en niveles significativamente más bajos que el control negativo (BV para 4D-T-EO y 4D-Af-EO *vs.* 4D-WATER = 26,27 ± 14,61% y 31,08 ± 16,52% *vs.* 51,35 ± 5,38%; *p*<0,001 para ambas comparaciones). El enjuague con T-EO mostró una actividad bactericida ligeramente mayor que la solución de Af-EO, aunque no se lograron resultados significativos. Con respecto a la BV por capas, las capas superficiales presentaron una BV significativamente mayor que las capas

profundas en todos los experimentos. Las formulaciones de T-EO y Af-EO fueron significativamente más eficaces a la hora de reducir el BV de las capas 1 y 2 que el control negativo, permaneciendo la capa 3 inalterada (BV capa 1 = 40.10 ± 17.31% y 39.81 ± 19.09% *vs.* 82.47 ± 7,58%; *p*<0,001, para ambas comparaciones. BV capa 2 = 24,32 ± 16,16% y 30,73 ± 17,06% *vs.* 51,76 ± 13,53%; *p*<0,001, para ambas comparaciones)

DISCUSIÓN:

En el presente estudio, el biofilm de 48 horas tuvo un grosor de 20 -22 µm, lo que coincide con los datos encontrados en otros estudios sobre el PL-biofilm formado *in situ* (21-27 µm) [10, 11, 23]. Después de un solo enjuague bucal, no se encontró ninguna reducción en el grosor del PL-biofilm con ninguna de las soluciones de EO. Este hallazgo es coherente con la literatura anterior con respecto a los T-EO [23]; sólo en el caso de la solución de CHX al 0,2% se pudieron detectar algunas ligeras reducciones [10].

Ambas soluciones de EO lograron una excelente actividad antibacteriana, con reducciones de BV del 57% y el 67%, alcanzando niveles de alrededor del 5% a 30 segundos post-enjuague. Después del alto efecto antibacteriano inmediato que tuvieron ambas formulaciones, la BV comenzó su lenta recuperación, aunque más gradualmente en el caso de la solución de T-EO.

En un estudio previo de nuestro grupo de investigación, en el que se comparó la actividad antibacteriana de T-EO con el 0,2% CHX, se detectó que el antiséptico T-EO tenía un efecto antibacteriano aún mayor que el CHX al 0,2% (Objetivo 1). De hecho, el enjuague T-EO tuvo un BV inmediato cercano a cero (fue alrededor del 1%) comparado con el 5% para la CHX. En ese estudio, el enjuague T-EO también mantuvo la BV bajo los niveles basales hasta 7 horas después del enjuague (Objetivo 1). Por el contrario, otros estudios encontraron que las soluciones de T-EO no eran tan eficaces como se describe en este documento. En este sentido, Gosau et al. [24] observaron que la BV después de una sola aplicación de T-EO era de aproximadamente el 20%.

Los dos enjuagues de EO fueron eficaces a la hora de reducir el grosor del biofilm formado después de 4 días en comparación con el control

negativo. De hecho, el grosor medio después de la aplicación de la solución de Af-EO fue casi tres veces menor que el del agua estéril (7,9 µm frente a 22,8 µm); en el caso de la solución de T-EO, el grosor medio obtenido fue menos de la mitad que para el agua estéril (9,9 µm frente a 22,8 µm). Estos resultados concuerdan con investigaciones previas sobre los T-EO y otros antisépticos [42, 46]. En un estudio anterior, los investigadores encontraron un grosor de 10 µm después de 4 días de la utilización continua de T-EO (Objetivo 2). Jentsch et al. [46], utilizando un microscopio electrónico de barrido, obtuvieron un grosor de 10,5 µm después de 3 días de la utilización diaria de T-EO.

Según el conocimiento del autor, sólo hay un estudio sobre el CG en un PL-biofilm de 4 días *in situ* (Objetivo 2). Nuestros resultados concuerdan con este estudio, ya que el CG para el control negativo fue casi el mismo (alrededor del 73-75%) y fue ligeramente mejor para el enjuague de T-EO (47% *vs.* 54%). Las soluciones de EO fueron, sin embargo, menos eficaces que la CHX al 0,2%, que tuvo un CG de un 20% en el mismo período. No se encontraron resultados publicados para los Af-EO, que en la presente serie obtuvieron un CG de un 33% y siendo más eficaces que la solución de T-EO a la hora de condicionar este parámetro microscópico.

En la presente serie, la BV del PL-biofilm de 4 días se redujo con las soluciones de T-EO y Af-EO (49% y 40%, respectivamente, en comparación con el control negativo). No se han encontrado otros estudios en relación con el Af-EO, pero los enjuagues de T-EO han demostrado previamente tener un elevado efecto antiplaca en términos de reducción de BV del PL-biofilm después de 4 días de uso, alcanzando niveles de reducción cercanos a los de 0,2% de CHX (74% frente a 77%) (Objetivo 2).

Los mejores resultados microscópicos en la presente serie del enjuague de Af-EO en relación con el CG y el grosor pueden explicarse por una teoría dual. La composición de los dos enjuagues difiere, aparte de en el etanol, en que el lauril sulfato sódico está presente en el Listerine® Zero™, pero no en el Mentol™. Este componente ha demostrado ser eficaz en la reducción de la BV [50-52] y la formación de placa [53, 54]. Su efecto antibacteriano puede deberse a la formación de poros en las membranas bacterianas, lo que podría aumentar la fluidez de la membrana, reducir las

cadenas de fosfolípidos en la membrana, aumentar el movimiento de rotación de las moléculas lipídicas y cambiar la distribución lateral de las proteínas y los lípidos de la membrana [51]. Su efecto sobre la placa dental puede deberse a la pérdida de partículas de alta densidad presentes en la matriz celular.

En cuanto al otro elemento diferenciador, el etanol, sus efectos sobre el biofilm se estudiaron extensamente en los años noventa. Se observó que las bacterias presentes en el biofilm se adaptaban fisiológicamente y se volvían más resistentes al estrés, incluyendo el inducido por los agentes antimicrobianos [55, 56].

De hecho, en estudios anteriores, un aumento en el crecimiento de placa *in situ* se describió en un modelo de 4 días después de que los voluntarios se enjuagasen dos veces al día con una formulación que contenía un 50% de etanol [57]. Sissons et al. [56] reportaron que las concentraciones de alcohol entre un 20% y un 30% producían inicialmente una inactivación rápida de las bacterias presentes en el biofilm, pero rápidamente perdían su actividad y una gran población resistente permanecía sin cambios.

En el mismo sentido, estudios de largo plazo [58] aparecen en la literatura comparando el uso de los EO que contienen etanol con un control negativo de agua y su vehículo de disolución (alcohol al 26,9% y el resto de los excipientes). Aunque los T-EO tuvieron un efecto antiplaca significativo después de 9 meses de uso continuo, el vehículo de disolución produjo un aumento del 7,3% en los niveles de placa después de este período de aplicación.

CONCLUSIONES

1. Un único enjuague de la formulación tradicional de aceites esenciales presentó un efecto antibacteriano inmediato muy elevado *in situ* y una sustantividad que perduró al menos 7 horas después de su aplicación sobre el biofilm de placa dental de 2 días no desestructurado. Estos resultados fueron incluso mejores que los observados con la clorhexidina al 0,2% en las mismas condiciones.

En consecuencia, un único enjuague de aceites esenciales con alcohol es una medida eficaz contra el biofilm oral, representando una buena alternativa a la clorhexidina, como un enjuague preoperatorio, en los procedimientos periodontales o complemento en el post-tratamiento.

2. En un modelo *in situ* de biofilm de 4 días no desestructurado, la fórmula tradicional a base de aceites esenciales presentó un elevado efecto antiplaca. Los aceites esenciales desarrollaron una actividad antibacteriana muy alta y similar a la detectada con la clorhexidina al 0,2%, aunque ésta muestra mejores resultados en la reducción del grosor y área ocupada por el biofilm de placa dental.

En consecuencia, la solución tradicional de aceites esenciales es una alternativa fiable a la clorhexidina para el control del biofilm oral y prevención de sus efectos secundarios cuando se utiliza de forma continua.

3. En un modelo *in situ* de biofilm de placa dental de 2 días no desestructurado, después de un único enjuague, ambas formulaciones de aceites esenciales (con y sin alcohol) presentaron una actividad antibacteriana inmediata muy alta y una sustantividad que dura al menos 7 horas después de una única aplicación. En un modelo *in situ* de biofilm de placa dental de 4 días no desestructurado, ambas formulaciones de aceites esenciales demostraron un elevado efecto antiplaca, aunque la fórmula sin alcohol mejoró en la reducción del grosor del biofilm y del área ocupada.

En consecuencia, la solución de aceites esenciales sin alcohol representa una opción fiable como agente antibacteriano y antiplaca para el control del biofilm oral.

REFERENCIAS

1. **Overman, P.R.**, *Biofilm: a new view of plaque.* J Contemp Dent Pract, 2000. 1(3): p. 18-29.

2. **Marsh, P.D.**, *Microbiologic aspects of dental plaque and dental caries.* Dent Clin North Am, 1999. 43(4): p. 599-614, v-vi.

3. **Newman, H.N. and Wilson, M.A.**, *Dental plaque revisited : oral biofilms in health and disease : proceedings of a conference held at the Royal College of Physicians*. 1999, Cardiff: Bioline.

4. **Auschill, T.M., Hellwig, E., Sculean, A., Hein, N., and Arweiler, N.B.**, *Impact of the intraoral location on the rate of biofilm growth.* Clin Oral Investig, 2004. 8(2): p. 97-101.

5. **Al-Ahmad, A., Wunder, A., Auschill, T.M., Follo, M., Braun, G., Hellwig, E., and Arweiler, N.B.**, *The in vivo dynamics of Streptococcus spp., Actinomyces naeslundii, Fusobacterium nucleatum and Veillonella spp. in dental plaque biofilm as analysed by five-colour multiplex fluorescence in situ hybridization.* J Med Microbiol, 2007. 56(Pt 5): p. 681-7.

6. **Hannig, C. and Hannig, M.**, *The oral cavity--a key system to understand substratum-dependent bioadhesion on solid surfaces in man.* Clin Oral Investig, 2009. 13(2): p. 123-39.

7. **Palmer, R.J., Jr., Wu, R., Gordon, S., Bloomquist, C.G., Liljemark, W.F., Kilian, M., and Kolenbrander, P.E.**, *Retrieval of biofilms from the oral cavity.* Methods Enzymol, 2001. 337: p. 393-403.

8. **Costerton, J.W., Stewart, P.S., and Greenberg, E.P.**, *Bacterial biofilms: a common cause of persistent infections.* Science, 1999. 284(5418): p. 1318-22.

9. **Cousido, M.C., Tomas Carmona, I., Garcia-Caballero, L., Limeres, J., Alvarez, M., and Diz, P.**, *In vivo substantivity of 0.12% and 0.2% chlorhexidine mouthrinses on salivary bacteria.* Clin Oral Investig, 2010. 14(4): p. 397-402.

10. **García-Caballero, L., Quintas, V., Prada-López, I., Seoane, J., Donos, N., and Tomás, I.**, *Chlorhexidine substantivity on salivary flora and plaque-like biofilm: an in situ model.* PLoS One, 2013. 8(12): p. e83522.

11. **Prada-López, I., Quintas, V., Donos, N., Suárez-Quintanilla, D., and Tomás, I.**, *Characteristics of in situ oral biofilm after 2 and 4 days of evolution.* Quintessence Int, 2015. 46 (4): p. 287-298.

12. **Tomás, I., Cousido, M.C., García-Caballero, L., Rubido, S., Limeres, J., and Diz, P.**, *Substantivity of a single chlorhexidine mouthwash on salivary flora: influence of intrinsic and extrinsic factors.* J Dent, 2010. 38(7): p. 541-6.

13. **Tomás, I., García-Caballero, L., Cousido, M.C., Limeres, J., Álvarez, M., and Diz Dios, P.**, *Evaluation of chlorhexidine substantivity on salivary flora by epifluorescence microscopy.* Oral Dis, 2009. 15(6): p. 428-33.

14. **Tomás, I., García-Caballero, L., López-Álvar, E., Suárez, M., Diz, P., and Seoane, J.**, *In situ chlorhexidine substantivity on saliva and plaque-like biofilm: influence of circadian rhythm.* J Periodontol, 2013. 84(11): p. 1662-72.

15. **Quintas, V., Prada-López, I., Donos, N., Suárez-Quintanilla, D., and Tomás, I.**, *In situ neutralisation of the antibacterial effect of 0.2% chlorhexidine on salivary microbiota: quantification of substantivity.* Arch Oral Biol, 2015. 60(8): p. 1109-16.

16. **Prada-López, I., Quintas, V., Casares-De-Cal, M.A., Suárez-Quintanilla, J.A., Suárez-Quintanilla, D., and Tomás, I.**, *Ex vivo vs. in vivo antibacterial activity of two antiseptics on oral biofilm.* Front Microbiol, 2015. 6: p. 655.

17. **Prada-López, I., Quintas, V., and Tomás, I.**, *The intraoral device of overlaid disk-holding splints as a new in situ oral biofilm model.* J Clin Exp Dent, 2015. 7(1): p. e126-32.

18. **Fuchslocher Hellemann, C., Grade, S., Heuer, W., Dittmer, M.P., Stiesch, M., Schwestka-Polly, R., and Demling, A.P.**, *Three-dimensional analysis of initial biofilm formation on polytetrafluoroethylene in the oral cavity.* J Orofac Orthop, 2013.

19. **Tawakoli, P.N., Al-Ahmad, A., Hoth-Hannig, W., Hannig, M., and Hannig, C.**, *Comparison of different live/dead stainings for detection and quantification of adherent microorganisms in the initial oral biofilm.* Clin Oral Investig, 2013. 17(3): p. 841-50.

20. **Hannig, C., Basche, S., Burghardt, T., Al-Ahmad, A., and Hannig, M.**, *Influence of a mouthwash containing hydroxyapatite microclusters on bacterial adherence in situ.* Clin Oral Investig, 2013. 17(3): p. 805-14.

21. **Hannig, C., Kirsch, J., Al-Ahmad, A., Kensche, A., Hannig, M., and Kummerer, K.**, *Do edible oils reduce bacterial colonization of enamel in situ?* Clin Oral Investig, 2013. 17(2): p. 649-58.

22. **Filoche, S.K., Coleman, M.J., Angker, L., and Sissons, C.H.**, *A fluorescence assay to determine the viable biomass of microcosm dental plaque biofilms.* J Microbiol Methods, 2007. 69(3): p. 489-96.

23. **Dong, W.L., Zhou, Y.H., Li, C.Z., Liu, H., Shang, S.H., and Pan, B.Q.**, *[Establishment and application of an intact natural model of human dental plaque biofilm].* Shanghai Kou Qiang Yi Xue, 2010. 19(2): p. 196-201.

24. **Gosau, M., Hahnel, S., Schwarz, F., Gerlach, T., Reichert, T.E., and Burgers, R.**, *Effect of six different peri-implantitis disinfection methods on in vivo human oral biofilm.* Clin Oral Implants Res, 2010. 21(8): p. 866-72.

25. **van der Mei, H.C., White, D.J., Atema-Smit, J., van de Belt-Gritter, E., and Busscher, H.J.**, *A method to study sustained antimicrobial activity of rinse and dentifrice components on biofilm viability in vivo.* J Clin Periodontol, 2006. 33(1): p. 14-20.

26. **Marsh, P.D. and Bradshaw, D.J.**, *Microbiological effects of new agents in dentifrices for plaque control.* Int Dent J, 1993. 43(4 Suppl 1): p. 399-406.

27. **ten Cate, J.M. and Marsh, P.D.**, *Procedures for establishing efficacy of antimicrobial agents for chemotherapeutic caries prevention.* J Dent Res, 1994. 73(3): p. 695-703.

28. **Newman, H.N.**, *Art and science, culture and dental research.* J Dent Res, 1996. 75(1): p. 526-7.

29. **Manau Navarro, C. and Guasch Serra, S.**, *Métodos de control de la placa bacteriana*, in *Odontología preventiva y comunitaria: Principios, métodos y aplicaciones*, E. Cuenca Sala, C. Manau Navarro, and L. Serra Majem, Editors. 2003: Barcelona. p. 79.

30. **Axelsson, P.,** *Preventive Materials, Methods, and Programs*. 2004, Quintessence: Surrey, U.K. p. 184.

31. **Vlachojannis, C., Winsauer, H., and Chrubasik, S.**, *Effectiveness and safety of a mouthwash containing essential oil ingredients.* Phytother Res, 2013. 27(5): p. 685-691.

32. **Stoeken, J.E., Paraskevas, S., and van der Weijden, G.A.**, *The long-term effect of a mouthrinse containing essential oils on dental plaque and gingivitis: a systematic review.* J Periodontol, 2007. 78(7): p. 1218-28.

33. **Carson, C.F., Mee, B.J., and Riley, T.V.**, *Mechanism of action of Melaleuca alternifolia (tea tree) oil on Staphylococcus aureus determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy.* Antimicrob Agents Chemother, 2002. 46(6): p. 1914-20.

34. **Fine, D.H.**, *Mouthrinses as adjuncts for plaque and gingivitis management. A status report for the American Journal of Dentistry.* Am J Dent, 1988. 1(6): p. 259-63.

35. **McCullough, M.J. and Farah, C.S.**, *The role of alcohol in oral carcinogenesis with particular reference to alcohol-containing mouthwashes.* Aust Dent J, 2008. 53(4): p. 302-5.

36. **La Vecchia, C.**, *Mouthwash and oral cancer risk: an update.* Oral Oncol, 2009. 45(3): p. 198-200.

37. **Gilbert, P. and Moore, L.E.**, *Cationic antiseptics: diversity of action under a common epithet.* J Appl Microbiol, 2005. 99(4): p. 703-15.

38. **Prada-López, I., Quintas, V., Vilaboa, C., Suárez-Quintanilla, D., and Tomás, I.**, *Devices for in situ development of non-disturbed oral biofilm. a systematic review.* Front Microbiol, 2016. 7: p. 1055.

39. **Sliepen, I., Van Essche, M., Quirynen, M., and Teughels, W.**, *Effect of mouthrinses on Aggregatibacter actinomycetemcomitans biofilms in a hydrodynamic model.* Clin Oral Investig, 2010. 14(3): p. 241-50.

40. **Pan, P., Barnett, M.L., Coelho, J., Brogdon, C., and Finnegan, M.B.**, *Determination of the in situ bactericidal activity of an essential oil mouthrinse using a vital stain method.* J Clin Periodontol, 2000. 27(4): p. 256-61.

41. **Fine, D.H., Furgang, D., Sinatra, K., Charles, C., McGuire, A., and Kumar, L.D.**, *In vivo antimicrobial effectiveness of an essential oil-containing mouth rinse 12 h after a single use and 14 days' use.* J Clin Periodontol, 2005. 32(4): p. 335-40.

42. **Jentsch, H., Hombach, A., Beetke, E., and Jonas, L.**, *Quantitative transmission electron microscopic study of dental plaque--an in vivo study with different mouthrinses.* Ultrastruct Pathol, 2002. 26(5): p. 309-13.

43. **Arweiler, N.B., Lenz, R., Sculean, A., Al-Ahmad, A., Hellwig, E., and Auschill, T.M.**, *Effect of food preservatives on in situ biofilm formation.* Clin Oral Investig, 2008. 12(3): p. 203-8.

44. **Gu, H., Fan, D., Gao, J., Zou, W., Peng, Z., Zhao, Z., Ling, J., and LeGeros, R.Z.**, *Effect of ZnCl2 on plaque growth and biofilm vitality.* Arch Oral Biol, 2012. 57(4): p. 369-75.

45. **Auschill, T.M., Hein, N., Hellwig, E., Follo, M., Sculean, A., and Arweiler, N.B.**, *Effect of two antimicrobial agents on early in situ biofilm formation.* J Clin Periodontol, 2005. 32(2): p. 147-52.

46. **Jentsch, H., Mozaffari, E., and Jonas, L.**, *Scanning electron microscopy of growing dental plaque: a quantitative study with different mouth rinses.* Ultrastruct Pathol, 2013. 37(4): p. 233-40.

47. **Al-Ahmad, A., Wiedmann-Al-Ahmad, M., Auschill, T.M., Follo, M., Braun, G., Hellwig, E., and Arweiler, N.B.**, *Effects of commonly used food preservatives on biofilm formation of Streptococcus mutans in vitro.* Arch Oral Biol, 2008. 53(8): p. 765-72.

48. **Rosin, M., Welk, A., Kocher, T., Majic-Todt, A., Kramer, A., and Pitten, F.A.**, *The effect of a polyhexamethylene biguanide mouthrinse compared to an essential oil rinse and a chlorhexidine rinse on bacterial counts and 4-day plaque regrowth.* J Clin Periodontol, 2002. 29(5): p. 392-9.

49. **Ulkur, F., Arun, T., and Ozdemir, F.**, *The effects of three different mouth rinses in a 4-day supragingival plaque regrowth study.* Eur J Dent, 2013. 7(3): p. 352-8.

50. **Jenkins, S., Addy, M., and Newcome, R.**, *Triclosan and sodium lauryl sulphate mouthrinses. (II). Effects of 4-day plaque regrowth.* J Clin Periodontol, 1991. 18(2): p. 145-8.

51. **Petersen, F.C., Assev, S., and Scheie, A.A.**, *Combined effects of NaF and SLS on acid- and polysaccharide-formation of biofilm and planktonic cells.* Arch Oral Biol, 2006. 51(8): p. 665-71.

52. **Ledder, R.G., Sreenivasan, P.K., DeVizio, W., and McBain, A.J.**, *Evaluation of the specificity and effectiveness of selected oral hygiene actives in salivary biofilm microcosms.* J Med Microbiol, 2010. 59(Pt 12): p. 1462-8.

53. **Waaler, S.M., Rolla, G., Skjorland, K.K., and Ogaard, B.**, *Effects of oral rinsing with triclosan and sodium lauryl sulfate on dental plaque formation: a pilot study.* Scand J Dent Res, 1993. 101(4): p. 192-5.

54. **Robinson, C., Strafford, S., Rees, G., Brookes, S.J., Kirkham, J., Shore, R.C., Watson, P.S., and Wood, S.**, *Plaque biofilms: the effect of chemical environment on natural human plaque biofilm architecture.* Arch Oral Biol, 2006. 51(11): p. 1006-14.

55. **Anwar, H., Strap, J.L., and Costerton, J.W.**, *Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy.* Antimicrob Agents Chemother, 1992. 36(7): p. 1347-51.

56. **Sissons, C.H., Wong, L., and Cutress, T.W.**, *Inhibition by ethanol of the growth of biofilm and dispersed microcosm dental plaques.* Arch Oral Biol, 1996. 41(1): p. 27-34.

57. **Gjermo, P., Baastad, K.L., and Rolla, G.**, *The plaque-inhibiting capacity of 11 antibacterial compounds.* J Periodontal Res, 1970. 5(2): p. 102-9.

58. **Gordon, J.M., Lamster, I.B., and Seiger, M.C.**, *Efficacy of Listerine antiseptic in inhibiting the development of plaque and gingivitis.* J Clin Periodontol, 1985. 12(8): p. 697-704.

GLOSSARY OF ABBREVIATIONS

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GLOSSARY OF ABBREVIATIONS

3-D: 3-dimensional

4D-T-EO: 4 days of daily mouthwashes with the traditional formula of essential oils

4D-0.2% CHX: 4 days of daily mouthwashes with 0.2% chlorhexidine

4D-WATER: 4 days of daily mouthwashes with sterile water

Af-EO: alcohol-free essential oils

CHX: chlorhexidine

CLSM: confocal laser scanning microscope

DAPI: 4'6-diamidino-2-phenylidole, diacetate

DPSS: diode-pumped solid-state

EO: essential oils

EtBr: ethidium bromide

FDA: fluorescein di-acetate

FISH: fluorescent *in situ* hybridization

IDODS: intraoral device of overlaid disk-holding splints

M-0.2% CHX: mouthwash with 10 mL of 0.2% CHX

M-Af-EO: mouthwash with 20 mL of alcohol-free essential oils

M-T-EO: mouthwash with 20 mL of traditional essential oils

PI: propidium iodide

PL-biofilm: plaque-like biofilm

PMT: photomultiplier

SEM: scanning electron microscopy

TEM: transmission electron microscopy

T-EO: traditional formulation of essential oils

VBNC: viable but non-culturable

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INTRODUCTION

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I.1. BACTERIA AND BIOFILM

The bacteria are prokaryotic single-cell microorganisms. They are the predominant form of life on Earth since they are ubiquitous, very heterogeneous and can adapt to lots of changes in their environment. For all these reasons and many others, bacteria are recognised to be virtually unextinguishable. In nature, it is estimated that over 95% of bacteria live in their natural habitats, the biofilms [1]. The formation of a biofilm on an aquatic system satisfies three indispensable conditions: 1. The metabolically active bacteria show a great capacity to adhere to the surfaces. 2. The extent of biofilm is controlled by a number of nutrients available for cell duplication and exopolysaccharide matrix formation. 3. Bacteria do not adhere to surfaces when the ecosystem is poor in nutrients [2].

Biofilms are, as well as bacteria, ubiquitous. They can form on any natural and artificial surface immersed in aqueous environments: the river and sea rocks, the hull of the boats, the contact lenses, the surfaces of the teeth… [3]. Today, we know that biofilms represent the preferred form of association by bacteria, as they provide a safer environment [4] (Figure I.1). In fact, biofilms are organised to maximise energy, spatial arrangements, communication, and continuity of the community of microorganisms. At the same time, they provide protection and may help the bacteria to survive and grow in hostile environments with common presence of toxic compounds. As a result, biofilm formation may, therefore, be a survival mechanism for bacteria and other microbes living in an aquatic environment [5]. It has been found that this complex structure makes the biofilm bacteria from 10 to 1000 times more resistant to an antimicrobial agent than the planktonic cells [6].

It was not until 1975 when the word “biofilm” first appeared in a scientific publication [7]. Years later, in 1990, the United States National Science Foundation founded the Centre for Biofilm Engineering at Montana State University in Bozeman. Since that time, the field of biofilm research has exploded. New tools and techniques are continually pioneered to help understand the secrets of microbial community interactions.

Donlan and Costerton [8] defined biofilm as *“a microbially derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other. These bacteria are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.*” This definition, unlike the previous ones, which did not take into account the biofilm phenotype establishes the properties of the biofilm and therefore is considered more complete. Bacterial populations that form a matrix and grow on a surface do not necessarily constitute a biofilm. For example, bacteria growing on an agar plate behave as planktonic cells stranded on a surface and show none of the inherent characteristics of true biofilms.

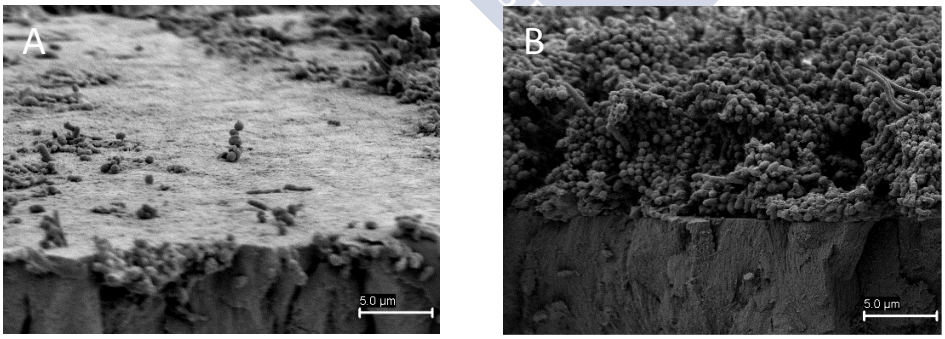


Figure I.1. Oral biofilm formed on an enamel disk. A) Image of the surface of the disk just some minutes after its exposure to the oral cavity. Note how bacteria start to attach one to another. B) Image of a 48-hour biofilm. Note how the bacteria grow attached one to another, creating a protective environment. Images were taken by scanning electron microscope (SEM).

I.2 THE ORAL BIOFILM

In the oral cavity, a tremendous amount of microbial diversity has been found. Approximately, 600 to 1000 bacterial species have been identified as permanent or transient microbiota inside the mouth [9, 10]. Since there are a large variety and concentration of microbes in a small space, all types of interactions have been developed over thousands of years of coevolution. These relationships are mutualistic, synergistic and facilitate cohabitation on oral surfaces taking advantage of metabolic by-products. Conversely, in other cases, these relationships are competitive or antagonistic [11].

In the mouth, biofilms develop spontaneously on the surfaces of teeth, prostheses, dental implants and oral epithelium [3]. Due to differences in the ecological niches inherent to the various intraoral locations, the microbiota that colonises the mucosal surface differs in composition from that of dental plaque [12]. This is defined as a complex microbial community that grows as a biofilm on the surface of the tooth enamel [13]. Dental plaque is considered a specialised model of microbial biofilm, similar to that developed on different surfaces in many systems with an aquatic environment [14]. The compact arrangement of bacteria makes it one of the most promising ecological niches for analysing both the behaviour as well as the biological and physiological properties of biofilm [3]. In this sense, it has been shown that the behaviour of dental plaque corresponds to that of a classic biofilm [15, 16] and that many of its properties are common to any other existing biofilm in nature [17, 18].

The dental plaque probably represents the most studied biofilm expression of all that has been described in the human being. In the seventeenth century a textile merchant named Antoni van Leeuwenhoek

first observed "animalcules" in the organic remains that covered the surface of his teeth (Figure I.2). Until this moment, it was believed that these deposits adhered to the dental surfaces consisted of, mostly, food debris. Two centuries after, Black [19] defined these deposits as “gelatinous microbial plaques” on teeth. Afterwards, in the twentieth century, these “microbial plaques” were associated with oral diseases [20], and in 1976, Marshall described the involvement of very thin fibres of extracellular polymers that anchored the bacteria to the surfaces [21].

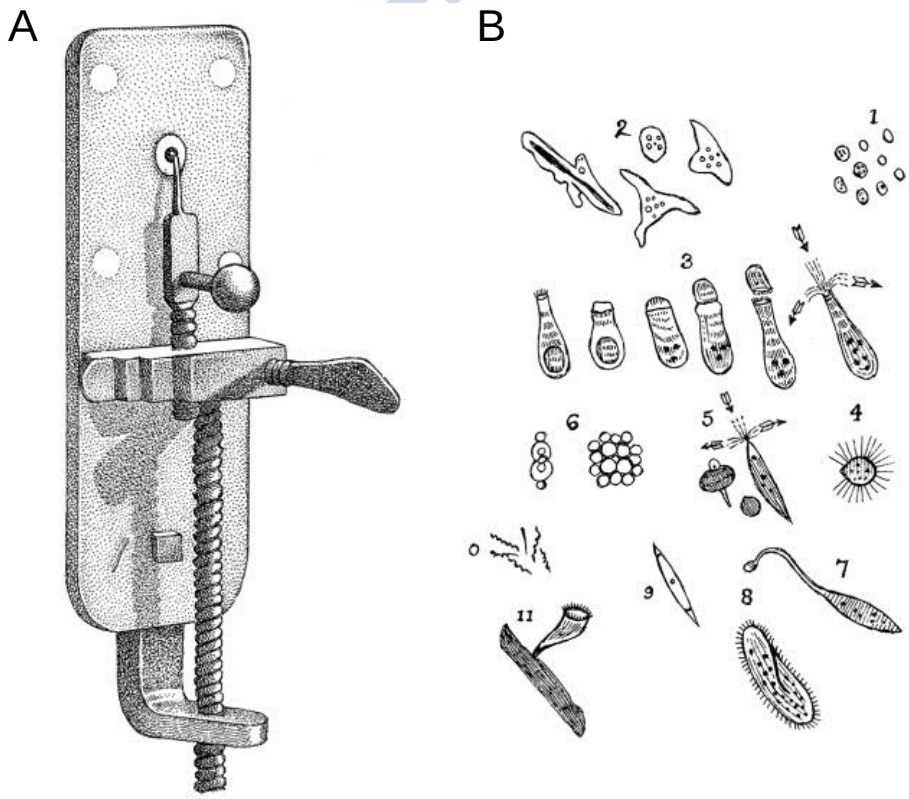


Figure I.2. A) Drawing of one of the microscopes designed by Antoni van Leeuwenhoek. B) “Animalcules” drawn by Antoni van Leeuwenhoek.

Nowadays, dental plaque has been widely known to be a specialised model of oral biofilm. It has been defined as a community of microorganisms found on the tooth surface and embedded in a matrix of polymers of salivary and bacterial origin [22].

I.3. STAGES OF BIOFILM FORMATION

In the colonisation of tooth surfaces and oral tissues, not all bacteria are attached at the same time. It occurs in a temporal manner being one species which becomes the scaffolding to which other species may adhere [11]. As a result, the composition of first colonisers will determine the later, giving importance to the action on the first stages of microbial colonisation.

Three stages can be characterised in the growth and development of the biofilm: initial adherence and lag phase, rapid growth, and steady state/detachment (Figure I.3). Biofilm formation begins with the adherence of bacteria to a tooth surface, followed by a lag phase in which changes in genetic expression (phenotypic shifts) are observed. A period of rapid growth then occurs, and an exopolysaccharide matrix is produced. During the steady state, the biofilm reaches growth equilibrium. Surface detachment or acquisition of new bacteria may also occur.

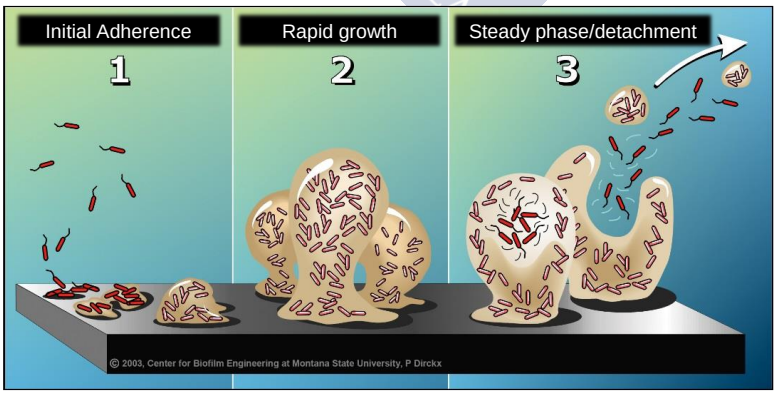


Figure I.3. Stages described in the formation of biofilm. The image was taken from Center for Biofilm Engineering at the Montana State University.

1. Initial Adherence and Lag Phase

The first phase of supragingival biofilm formation is the deposition of salivary components, known as acquired pellicle, on tooth surfaces. This pellicle makes the surface receptive to colonisation by specific bacteria. Salivary glands help in the formation of this acquired pellicle, producing a variety of proteins and peptides that further contribute to biofilm formation such as mucins, statherin and proline-rich proteins [23-25].

Acquired pellicle formation begins within a few minutes of a professional prophylaxis; within 1 hour or less, microorganisms attach to the pellicle [26-28]. Usually, Gram-positive cocci are the first microorganisms to colonise the teeth. As bacteria change from planktonic to sessile life, a phenotypic change in the bacteria occurs requiring significant genetic up-regulation (gene signalling that promotes this change). There is not too much bacterial growth since the genetic change is happening [29].

2. Rapid Growth

During this stage, the biofilm matrix is formed due to the large amounts of extracellular polysaccharides. The growth of microcolonies within the matrix occurs. As time passes by, additional varieties of bacteria adhere to the early colonisers – this process is known as coaggregation [30] - and the bacterial complexity of the biofilm increases.

These processes involve plenty of molecular interactions leading to complex three-dimensional (3-D) structural stratification within the biofilm. Coaggregation and subsequent cell division also increase the thickness of biofilm [31-33].

3. Steady State/Detachment

During the steady state phase, bacteria in the interior of biofilms slow their growth or become static. Bacteria deep within the biofilm show signs of death with disrupted bacterial cells and other cells without cytoplasm; bacteria near the surface remain intact. During this phase, crystals can be observed in the interbacterial matrix that may represent initial calculus mineralisation [34].

As already named, during the steady state stage, bacterial surface detachment occurs, with some bacteria travelling to form new biofilm colonies.

I.4. BIOFILM AND ORAL/GENERAL DISEASE

Biofilms are important because some resident species contribute to the maintenance of oral health and other species have the potential to cause local or systemic diseases [35]. Much of our interest in plaque (oral biofilm) is due to its potential to cause disease. Dental caries will not develop in the absence of plaque [36]. Plaque formed in the presence of dietary sucrose had much great acid-producing potential than that formed in the presence of monosaccharides [37]. However, in the absence of plaque, frequent rinsing with sucrose does not cause caries [38].

The relationship between plaque and gingivitis is clear-cut. When plaque is allowed to form on cleaned tooth surfaces associated with healthy gingivae, subclinical gingivitis develops within 3 days, and clinical gingivitis is detected between 10-21 days [39-42]. This gingivitis is reversible and will be completely resolved when plaque is eliminated by tooth brushing or rinsing with antiseptics [43]. Dental plaque is also associated with

periodontal diseases, halitosis [44] and in the regeneration, healing and cicatrisation of the tissues after oral manipulations [45].

The dental plaque accumulation is even more problematic when the body's immune system is compromised (leukaemia, agranulocytosis, HIV, etc.) since it can lead to the severe destruction of the periodontal and other tissues [35].

However, biofilms not only affect the development of oral diseases. They play an active role in infectious diseases within the body. Many infections are attributable to bacterial biofilms: otitis, conjunctivitis, osteomyelitis, cystic fibrosis, infective endocarditis and a big group of nosocomial infections that are related to dialysis catheters, prosthetic heart valves, tracheal intubation or internal devices.

I.5. THE ORAL BIOFILM STUDY

When studying a bacteria grown in a biofilm structure, we should take into account that the biofilm environment confers certain properties to bacteria that are not seen in their nomadic state. This fact justifies the importance of recognising dental plaque as a biofilm and not as bacteria in the planktonic state [46].

The study of bacteria in their natural habitat (a "biofilm") has evolved in the past few decades. While bacteria were originally studied in rich media and often in isolation from companion or competing species, after the discovering of the oral biofilm importance, its conditions were mimicked more closely in experiments. To this end, bacteria are grown together in consortia as they exist in the oral cavity. Models were developed for bacterial adherence and growth on solid surfaces rather than in pure liquid chemostats and with the simulation of the shear forces that bacteria

experience in the mouth. Growth media were developed to those which are compositionally similar in nutritional properties to dental plaque and saliva.

I.5.1. LIMITATIONS OF THE *IN VITRO* AND "DESTRUCTURED" *IN SITU* MODELS

I.5.1.1. *IN VITRO* MODELS

The development of *in vitro* biofilm models led to significant advances in the study of oral biofilms [47-51]. In fact, the first step in the study of complex oral bacterial communities was *in vitro* models based on simple designs. These simple models were used to analyse the structure and function of the biofilm [52], as well as evaluating the activity of different antimicrobials [53].

The ideal laboratory model should take into account of the following important features of the oral environment, which have a profound effect on the likely effectiveness of an antimicrobial agent *in vivo* [35]:

1. The biofilms form on a solid substratum (enamel, cementum, restorative materials, etc.) and are in contact with a thin film of liquid (saliva or gingival crevicular fluid).

2. The fluid is continually being replaced.

3. The bacteria are attached to the substratum by a conditioning film composed mainly of salivary glycoproteins or crevicular fluid proteins.

4. The biofilms are subjected to mechanical and hydrodynamic shear forces.

5. A wide variety of microorganisms is present in the biofilms.

6. The system may be aerobic or anaerobic.

The following are the most used *in vitro* models in the literature. They can be divided into two categories regarding the exchange of materials with the external environment (closed and open models):

Closed batch culture models

These are systems in which there is no exchange of materials (particularly nutrients and waste products) with the external environment. The system is constantly changing the terms of the concentration of these materials as well as physicochemical factors such as pH, redox potential, etc. They do not represent well an *in vivo* environment. However, they are technically simple to perform and are used for primary screening. Their representatives are mainly the agar plates and membrane filter-based models.

1. Growing on agar plates

Probably the simplest model, bacteria grow in an agar plate. The result is a biofilm that consists of an accumulation of cells firmly packaged and embedded in an extracellular matrix, but only representative of the biofilm in a solid-gas interphase (Figure I.4).

Figure I.4. Plate agar with sheep blood as culture media.

2. Membrane filter-based models

Instead of growing the bacteria directly on the agar, a biofilm grows in a membrane filter placed on the agar surface. After the biofilm growth, it can be withdrawn intact [54, 55].

Although these two systems are technically simple, they are poor models of dental plaques in their natural environment. The more noticeable defects of these models are:

1. The biofilms are produced at a solid/air interface rather than at a solid/liquid interface.
2. The substratum (agar) is inappropriate.
3. Nutrients are supplied to the biofilm via the biofilm/substratum interface rather than from the biofilm/fluid interface.
4. There is no simulation of salivary flow.

Open continuous culture models

These models involve the exchange of materials and microorganisms with the external environment so that fresh nutrients may be continually added while waste products are removed. The most representative models are the chemostat, the constant depth film fermenter and the artificial mouths.

1. Chemostat based models

These systems have a bioreactor to which fresh medium is continuously added. At the same time, culture liquid containing nutrients, metabolic products and microorganisms are continuously removed at the

same rate to keep the culture volume constant. In the oral biofilm study, it has been used for creating communities from up to 10 species of oral bacteria [56]. Afterwards, hydroxyapatite disks were used for multispecies biofilm grows over them [57].

2. Constant depth film fermenter

The most used model to generate reproducible biofilms that simulate dental plaque is composed by a glass recipient with stainless steel plaques, which are the holder of 15 wells. Each well has six cylindrical holes with plugs. These plugs can incorporate hydroxyapatite or enamel disks over which the biofilm grows. This system allows harvesting the biofilm without distorting its structure.

This system has been applied by numerous authors to study the oral biofilm [58-64], to evaluate the susceptibility to oral antiseptics of specific oral bacteria [55] and to generate a multispecies biofilm in similar conditions to the dental plaque [65].

3. Artificial mouths

Multiple artificial mouth systems could be employed for the long-term growth of multispecies plaque samples within a standardised, simulated oral environment generated by computer-controlled facilities. The basic purpose of artificial mouth is to imitate the oral environment with the help of “saliva substitutes”. The artificial mouth (Figure I.5) allows monitoring with precision the physical, chemical, biological and molecular characteristics of the biofilm [66]. Also, this system has been used to analyse the microbial interactions in dental plaque.

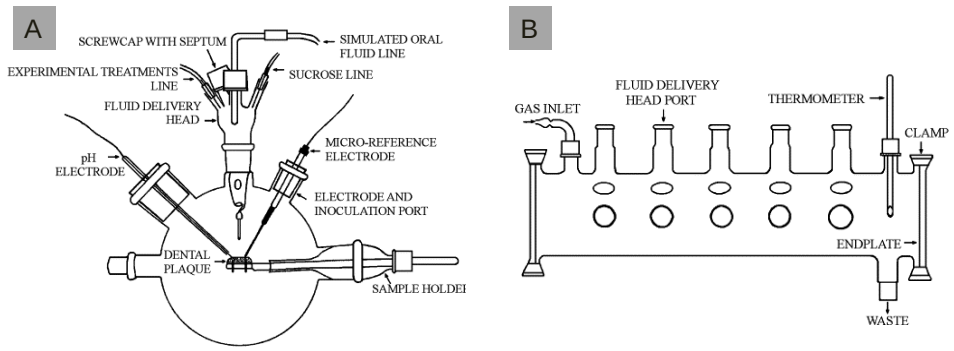


Figure I.5. Artificial mouth designed by Sissons et al. 2000 [67]. A) Cross-section of biofilm growth station. B) Longitudinal section of culture chamber. The images were taken from Tang et al. 2003 [66].

The creation of *in vitro* biofilm models has contributed significant breakthroughs in the study of oral diseases [47-51]. However, their known limitations have caused the scientific community recognises that the *in vitro* models might not generate a comparable biofilm to those found *in situ* [68-72]. For this reason, *in vitro* results must be interpreted cautiously [69, 72, 73]. This affirmation establishes the need to develop models of biofilm *in situ* which could be analysed *ex vivo* without distortion [4, 69, 73, 74].

On the other hand, the penetration of solutes during very brief periods of exposure (less than 120 seconds) has been the object of scarce research *in vitro* [71]. To date, biofilm models have been evaluated *in vitro* with ranges of exposure to antimicrobial agents ranging from several minutes [75] to hours [76], which has limited relevance to the oral cavity, in which the exposure to antimicrobial agents usually ranges from 30 seconds to 1 minute [77, 78].

I.5.1.2. *IN SITU* STUDIES OF "DISTURBED" ORAL BIOFILM

Provided all the limitations that *in vitro* studies have, many authors have assessed the dental plaque formed on the tooth surfaces *in situ* [79-87]. They have also evaluated the activity of different antiseptics on that *in situ* formed dental plaque by analysing the composition of the dental plaque by conventional culture methods [79-82]. However, the disadvantages of these culture microbiological methods are numerous and well-known [72, 88]. Fluorescence techniques have also been applied to study *in situ* the composition of dental plaque and the antibacterial effect of some antiseptics [83-86].

A common methodological characteristic of all these studies is that the evaluation of supragingival plaque is carried out on biological material previously extracted from the surface of the tooth (using cotton swabs or rolls, dental scalers…) [83-87, 89]. Therefore, the subgingival plaque is obtained by using paper tips or by mechanical debridement [80, 90], which is likely to disrupt the delicate 3-D relationship between cells, the extracellular matrix and the substrate [91, 92]. Another disadvantage of this type of studies is that the level of penetration of an antimicrobial agent in plaque samples cannot be evaluated since the samples are dispersed for analysis [85]. Consequently, this methodology provides an inadequate analysis of the architecture and *in situ* organisation of dental plaque, as well as the action of antimicrobial agents on its structure [71, 93].

To better understand the clinical effects of these agents in the interior of the biofilm, it is necessary to apply a methodology in which it grows directly inside the oral cavity and in which its 3-D structure is not disturbed with manipulation [91, 94].

I.5.2. *IN SITU* STUDIES OF UNDISTURBED ORAL BIOFILM

Not disturbing with manipulation means that the biofilm is not altered during its formation, recollection, processing or analysis. In the literature, this type of studies does not use natural teeth for collection, but disks of different materials that are introduced into the mouth for a variable period to expose them to the intraoral conditions of each individual. Therefore, it is not a dental plaque itself, but a biofilm presumably very similar to a dental plaque, which is generated under similar conditions and which sits on an artificial substrate, so that henceforth we will call it plaque-like biofilm (PL-biofilm) [95, 96]. The disks where the oral biofilm is formed are introduced into the oral cavity, supported by specially designed devices.

I.5.2.1. SUBSTRATES

The disks used as substratum, where the dental plaque is formed, have been made of different materials. These have ranged from human enamel and dentine, bovine enamel and dentine, glass, hydroxyapatite, titanium, dental restoration materials, regenerative membrane materials…

In some cases, the substrate has been defined by the objective of the study, i.e., if the aim is studying the peri-implantitis, the substrate will be more likely to be the titanium (with the different designed surfaces) [97-100].

In some others, authors have tried to apply materials which permit the obtaining of a presumably more similar biofilm to that formed on the tooth surface; for that reason, they used enamel and dentine (both human and bovine) [26, 27, 69, 70, 73, 84, 91, 93, 101-123]. There is also another group of studies that used hydroxyapatite [124-126], with the thought that a

substrate which is chemically more similar to the human enamel will provide a more trustful PL-biofilm.

In the nineties, the first studies using polished glass appeared [109]. This material was presented as the perfect substitute of enamel and hydroxyapatite, obtaining similar results regarding PL-biofilm viability and thickness but without the problems of autofluorescence that both enamel and hydroxyapatite have [96, 109]. In fact, it has been one of the most used substrates in the last decade [69, 70, 92, 93, 96, 127-131].

I.5.2.2. APPLIANCES

Once the different substrates have been already presented, another essential part of the process for the obtaining of an undisturbed oral biofilm is the appliance where the substrate is held during its time in the mouth. The first apparatus was designed in the seventies. It was a mandibular acrylic splint with enamel slides [132] (Figure I.6). This design was posteriorly used by the group of Nyvad in the eighties [101-103]. The evolution of this apparatus was made by Auschill et al. [69], who designed a maxillary appliance which combined metal and acrylic reducing the bulkiness.

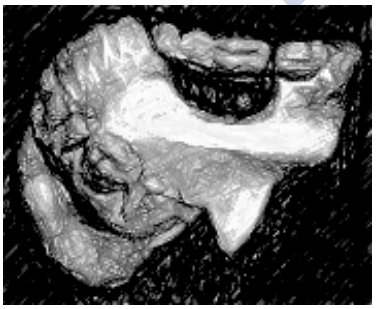


Figure I.6. Ahrens model. The image was taken from Ahrens, 1976 [132].

Other designed appliances have been the thermoplastic splints where the disks are attached with wax or silicone [133] (Figure I.7). There is also a variation that added metal to reinforce the thermoplastic soft splint [129].

Figure I.7. Hannig's device. The image was taken from Jung et al. 2010 [27].

One of the last designed apparatus has been the "intraoral device of overlaid disk-holding splints" (IDODS) [95, 96, 131] (Figure I.8). In this device, two splints of thermoplastic material (hard and soft ones) are used with the aim of interfering as little as possible with the normal life of the volunteer. The disks are self-retained, and no wax, adhesive or silicone is needed.

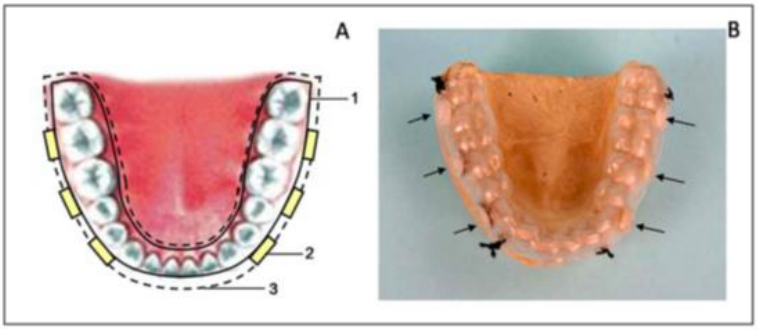


Figure I.8. IDODS scheme and over a cast. The images were taken from García-Caballero et al. 2013 [134].

Another used model has been the “Leeds *in situ* device” (Figure I.9), which is a ring that includes a substrate of human enamel [91, 110, 135, 136] and it adheres to buccal of first and second molars using a composite resin. From the designs presented, it is the only one which is not removable by the volunteer.

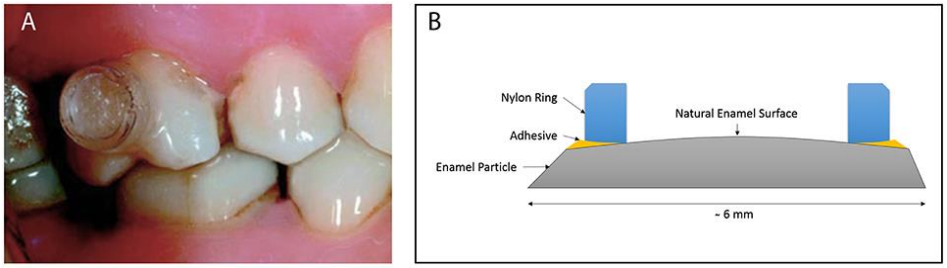


Figure I.9. Leeds *in situ* Device (LiD). A) Intraoral image by Pessan et al. 2008 [137]. B) Own design scheme.

Although all these designs can hold the substrates with more or less success and can seem useful to achieve the correct reproduction of the *in situ* oral biofilm, after their careful study [138], all of them show limitations at some point, such as at the aesthetic and hygienic levels or the biofilm growth environment. Some of them need specific teeth for retention or a specific pre-treatment of the tooth surface (etching and bonding) with the associated problem of its potential accidental unsticking [138]. From all of them, the IDODS seems to be the designed device with fewer disadvantages, for this reason, it was the one selected in the work of the present Thesis [138].

I.5.3. APPLICABILITY OF THE CONFOCAL LASER SCANNING MICROSCOPY

Over the years, different microscopy techniques have been applied to visualise the microstructure of the *in situ* PL-biofilm, including optical microscopy, transmission electron microscopy (TEM) and SEM [26, 139, 140]. With these techniques it is difficult to obtain images of the specimens in depth and, also, they require a processing that could cause retraction and loss of the biofilm matrix and produce artefacts in the samples [91, 141].

Fluorescence microscopy has become an essential tool in biology, particularly in the study of bacteria and their interactions in biofilms, owing to it has attributes that are not readily available in other optical microscopy techniques. Fluorescence illumination and observation are one of the most rapidly expanding microscopy techniques employed today, both in medical and biological sciences, which has spurred the development of more sophisticated microscopes and numerous fluorescence accessories. The advantages and disadvantages of the fluorescence microscopy are developed in the next paragraphs and summed in the Table I.1.

Table I.1. Advantages and disadvantages of the fluorescence microscopy.

FLUORESCENCE MICROSCOPY	
Advantages	*Disadvantages*
Study of live samples in their natural hydrated state	Lower power resolution than SEM or TEM
No fixation necessary—lower probability of artefacts	Problems with fluorescence in deep regions of thick specimens
Correction of the out-of-focus image*	Restricted conditions of the objective lenses
Possibility of observing micro-sections*	Fading and bleaching of fluorochromes
3-D image generation*	Laser intensity must be controlled
Images suitable for storage, display and processing	

**Only applicable to the CLSM.*

I.5.3.1. ADVANTAGES

One of the biggest advantages of fluorescence microscopy is the possibility to study live samples in their natural hydrated state (Figure I.10) [69, 93, 121] and the consequences that this brings with it. As previously stated, with SEM and TEM, the samples require preparation involving dehydration, fixing and embedding, which may cause disruptive shrinkage and loss of the biofilm matrix that can affect 73 to 98% of the *in situ* biofilm mass [141]. Furthermore, the time-consuming and tedious nature of the sectioning process for TEM constitutes a big drawback for this technique [142]. For all of these reasons, traditional electronic microscopy is prone to include artefacts in the sample that will affect mainly the delicate fluid-filled structures present in the biofilms [91].

Of the fluorescence microscopes, CLSM is the one that provides more advantages over conventional optical and electronic microscopes, and it

represents an evolution of epifluorescence or incident-light fluorescence. The most important feature of CLSM is the capability of isolating and collecting a plane of focus from within a sample, which is achieved by spatial filtering to eliminate out-of-focus light or flare in specimens that are thicker than the plane of focus [10]. In a fluorescence microscope, a small part of a sample may be in focus, but you look at the entire object (i.e. what is in focus is viewed as well as what is out of focus). The confocal microscope has a stepper motor attached to the fine focus, owing to this, it is possible to have a controllable depth of field, which provides the possibility to collect serial optical sections from thick specimens [72, 92, 121, 127, 143].

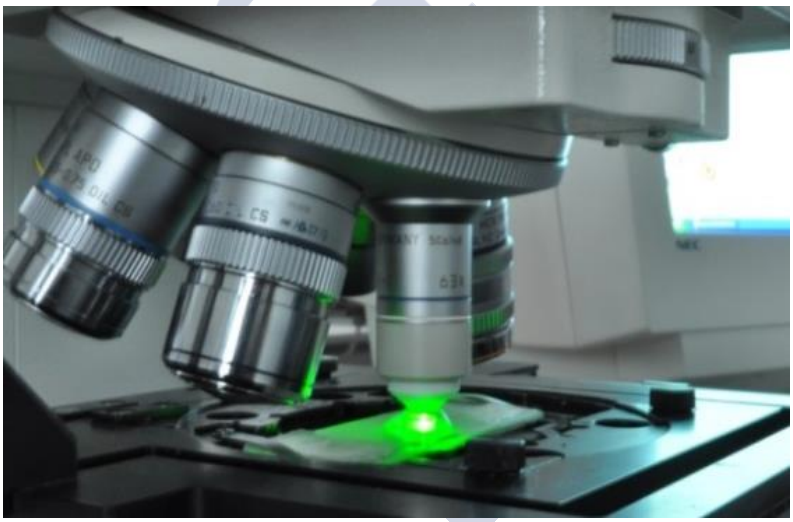


Figure I.10. The objective HCX APO L 63x/0.9 water-immersion lens during the analysis of a biofilm sample. Note the drop of water (shown in green, because of the exciting light) that keeps the sample hydrated (this photograph was taken in darkness).

The possibility of measuring serial optical sections allows for further capabilities of the system, such as the generation of 3-D images of living cells with submicron resolution, which are unique insofar as such spatial information can only be obtained from the hydrated material using the confocal technique [142]. These 3-D images are correlated, as far as

possible, with data obtained by the serial sectioning technique of hydrated biological specimens.

CLSM, with its various imaging modes, will prove invaluable for applications in the field of biology, both for its 3-D imaging capabilities and the possibility to observe live objects in their natural environment. In addition, the existence of sophisticated fluorescent probes permits the visualisation of the spatial organisation of specific substances inside the live biological material.

Although still lower than traditional electronic microscopes, CLSM has improved the resolving power with regard to other fluorescence microscopes, both transverse as well as along the optical axis. Furthermore, the manner in which data are collected in this instrument is suitable for storage, display and subsequent data processing using a digital computer.

I.5.3.2. DISADVANTAGES

For fluorescent light emitted by the deeper regions of the material, the incident and emitted photons have to travel through the more superficial layers of the sample. The thickness and the occasional heterogeneity of the crossed layers can act upon the value of the recorded signal, according to the two following processes. Firstly, the reflection, refraction and scattering of the incident beam by parts of the specimen located between the focal plane and the objective lens can, in principle, reduce the intensity of incident radiation (inner-filter effect). Secondly, the fluorescent photons can be attenuated by the same upper structures on their way back [144, 145]. Furthermore, an additional problem, in the case of thick biofilms, is the risk of underestimating bacterial numbers, owing to the degree of fading of the fluorochrome when multiple optical sections are acquired [127, 146].

Another limitation that should be taken into account relates to the optimal parameters of the objective lenses. These are designed to be operated under a very restricted set of conditions (refraction index of the immersion oil/water, the thickness of the cover glass, etc.). Deviations from the optimum nominal parameters can dramatically degrade the objective performance, resulting in some loss of image definition [147].

A further inconvenience is the bleaching of fluorochromes, which constitutes one of the most troublesome aspects that counteracts a quantitative fluorescence approach. Photobleaching is especially important in CLSM because the optical slices are performed sequentially. As a consequence, each slice has not received the same amounts of irradiation as its scan began [147].

The influence of laser power is associated with the photodestruction quantum yield, which directly depends on the intensity of the incident light. A laser power that is too high should not be used, as it can give rise to a bad signal-to-noise ratio. Additionally, an illumination that is too strong might damage biological structures, causing unwanted distortions in samples [147].

Other disadvantages that may be associated with fluorescence staining include: the existence of people (8–12% of males and almost 1% of females) that are green/red colour blind, who cannot extract any meaningful information from the images [61]; the influence of the type of bacteria (i.e., *Actinomyces* spp. often results in an irregular distribution of fluorescent signals possibly because of insufficient permeability of the bacterial cell walls) and of its physiological state (exponential-growth phase *vs.* stationary phase) about the staining properties [27, 148]; some fluorochromes showed

a significant non-specific binding to the matrix and great background fluorescence [149], or the detection of intermediate colours, the interpretation of which remains unclear [148].

I.5.3.3 RELIABILITY OF FLUORESCENCE TECHNIQUES AND MICROSCOPIES: COMPARISON OF BIOLOGICAL TECHNIQUES

Limitations of plate-culture techniques

Plating is a common quantification method that allows the determination of the number of culturable cells [150]. Although this technique has the advantage of determining the number of active bacteria, there are some disadvantages that could lead to misleading conclusions and can, at least, question its consideration as the *Gold Standard* (Table I.2). The traditional method for determining the number of microorganisms in a sample is the measurement of the viability by prior desorption through ultrasonication or vigorous agitation and subsequent plating onto different agar plates. Variations in this procedure may influence the results considerably [26], producing an underestimation of the number of cells if the dispersion is not performed accurately. Moreover, cells will not grow and form visible colonies if they are in a state of starvation or under stress [151, 152]. Furthermore, as this technique is both labour- and time-intensive, it is not suitable for high-throughput screening [150]. Besides, it needs the selection of certain species when studying multispecies biofilms from natural niches [8]. In addition, more than 700 bacterial species or phylotypes, of which over 50% have not been cultivated, have been detected in the oral cavity [9].

Table I.2. Summary of the main problems related to the plate-culture techniques.

MAIN DISADVANTAGES OF PLATE CULTURING
Desorption by ultrasonication, vortex, etc.
Impossibility of viability assessment without disrupting the biofilm structure
Cells do not grow if they are in starvation mode or are under stress
Laborious and time-consuming technique
Necessity of selection of certain species
50% of the bacterial species present in the oral cavity are not culturable

Overcoming plate-culture techniques with fluorescence techniques

All of the limitations of plate-culture techniques illustrate the necessity to develop methods other than culture plates for the visualisation, quantification and identification of bacteria [115]. One of these methods are the fluorescence-staining techniques followed by fluorescence microscopic analysis [153, 154]. Although fluorescence microscopic methods, such as fluorescence *in situ* hybridization (FISH), offer the opportunity to mark selected bacterial species or to stain all cells, there are more techniques that allow the differentiation between vital and dead bacteria [109, 115, 155].

- Fluorescence *in situ* hybridization

It has been shown that FISH, a technique that employs fluorescence-labelled, species-specific DNA probes, is a useful method for the detection of bacteria without too much disruption to their natural environment [72, 92, 113, 127, 128, 156]. Several authors have used FISH in combination with CLSM to obtain images of 3-D reconstructions of the natural microbiological environments of *in situ* oral biofilms, evaluating the levels of up to four different microorganisms in the biofilm formation after different periods of time [51, 72, 92, 113]. FISH permits the identification of non-culturable

bacteria and the faster development of new probes compared with the production and characterisation of antibodies [157].

However, one of the limitations of FISH is that it uses ribosomal RNA-targeted probes, and its sensitivity is, therefore, limited by the metabolic state of the bacteria present in the biofilm, as metabolically inactive microorganisms are considered to have a lower cellular ribosomal content [158]. Furthermore, FISH requires specimen fixation, including dehydration, potentially leading to similar problems to those observed with TEM and SEM, making it impossible to study the dynamic changes occurring in live biofilms [158, 159].

- Differentiation between vital and dead bacteria

In addition to the identification of species, the viability of the bacteria present in a biofilm is also of considerable interest. Several stages of viability are discussed and described in previously published reports: viable and culturable, viable but non-culturable (VBNC) dormant, non-viable and pre-lytic, and avital dead bacteria [155] (Table I.3.). The exact differentiation of these stages remains one of the greatest challenges in modern microbiology [155]. Typically, a combination of different dyes for vital and avital bacteria is used for this purpose. The interaction of different bacterial species with certain dyes differs distinctly, yielding different patterns of viability [155]. However, when investigating the efficacy of antibacterial rinses or reagents, it is of great relevance to visualise their effect on the viability of the oral biofilm because the bacteria present in biofilms are often much more resistant than microorganisms in the planktonic phase [12].

Table I.3. Stages of viability described in previously published reports, with their associated characteristics regarding their ability to form colony forming units (CFU) and the presence of metabolic activity and membrane integrity.

Bacterial State	*CFU*	*Metabolic Activity*	*Membrane Integrity*
Viable-vital and culturable	Yes	Yes	Yes
VBNC dormant	No	Yes	Yes
Non-viable and pre-lytic	No	No	Yes
Dead-avital	No	No	No

- Measurement of cell vitality

Cell vitality may be assessed by morphological changes or, more accurately, by changes in membrane permeability or physiological state, as inferred from the exclusion of certain dyes or the uptake and retention of others. Changes in morphology can be detected by forwarding and side-scattered characteristics, but this can be a crude measurement of vitality. On the contrary, differentiation by membrane permeability or the metabolic state offers the possibility to compare the percentage live/dead distribution of the bacteria, before and after treatment with different rinses or other adverse environmental conditions [160-162].

➢ Cell vitality based on membrane permeability

Many live/dead stains test the membrane integrity of respective bacteria. Most DNA-binding dyes are excluded by vital cells and can be used as viability dyes [163]. Many dyes used for the staining of non-viable bacteria enter cells passively and rapidly with damaged plasma membranes and bind to nucleic acids, yielding an enhancement in fluorescence [115].

One of the first staining assays for the detection of dead bacteria was ethidium bromide (EtBr). After the transport of the huge EtBr molecules into

dead cells with permeable membranes through the passive-diffusion processes, the molecules intercalate into DNA and show an enhancement in the fluorescence [164-166].

- Cell vitality based on functionality

Cell vitality determined by dye exclusion can give false information about the real health status of cells, especially when cells can exclude viability dyes but cannot proliferate or grow. Consequently, an assay on cell function is required to ensure detecting viable cells with the ability to proliferate [163].

Cell health can be measured by dyes that require a specific cell function, such as esterase activity in addition to an intact cell membrane. These dyes are usually non-fluorescent and are designed to enter healthy cells easily. Once inside cells, they are cleaved by esterase enzymes to generate a fluorescent product, which is retained in live cells with intact cell membranes, but leaks out of cells with compromised cell membranes. In this case, vital cells remain fluorescent and non-vital cells become non-fluorescent [163]. One of the first dyes used for the detection of vital cells was fluorescein diacetate (FDA), the uncoloured precursor of fluorescein. This dye develops its fluorescence after intracellular enzymatic metabolism by esterase [167] and accumulates in the cell, emitting green light after excitation with 490 nm light, theoretically, only in vital cells [167-169].

- Combination of fluorochromes

Many combinations of fluorochromes have been used to study oral biofilms since 1983, Netuschil et al. first used fluorescence to visualise dental plaque [167]. The combinations of both FDA/EtBr [70, 109, 121, 123,

170] and SYTO 9/propidium iodide (PI) [61, 85, 88, 95, 96, 98, 114-116, 129, 134, 171-178] have been the most employed, in addition to some studies that employed acridine orange [179], fluorescein alone [91] or DAPI [27].

- FDA/EtBr

Despite being the first and traditional combination used for this purpose, some problems have been found regarding the safety of FDA and EtBr as well as their ability to stain bacteria present in an oral biofilm reliably.

After the application of FDA, theoretically, only vital cells become fluorescent, although it has been shown to be rather unstable and it rapidly leaks from all cells, regardless of whether or not they are vital [155]. Loss of fluorescein occurs in intact cells quickly as a result of photobleaching and the diffusion of fluorescein across the cell membrane [180]. The use of FDA is, therefore, restricted to samples where it is not required to resolve cells nearby, and the time of exposure to illumination and the overall experiment must be short [180]. In comparison, carboxyfluorescein diacetate shows a longer intracellular accumulation, owing to its negative charge [155, 169]. Calcein acetoxymethyl, another fluorescent dye for vital cells, is readily cleaved by intracellular esterases to form fluorescent calcein. Once in this fluorescent state, calcein is distributed throughout the whole cell, nuclei and mitochondria [181]. Another valid alternative to FDA could be SYTO 9, which stains vital bacteria green without the problems of leakage and photobleaching that FDA has, although its staining mechanism is based on the intercalation of DNA and RNA rather than intracellular esterases [180].

The main problem of EtBr, however, is its well-known toxicity, which has led to the development of alternative reagents [28]. The results obtained

with PI are superior to EtBr, as it only penetrates damaged cells whereas EtBr may also stain vital bacteria [155]. Furthermore, EtBr is less reliable than PI, as the latter has a lower charge and, therefore, a higher affinity for DNA [182, 183].

Although the poor correlation was found between FDA/EtBr staining and CFU when investigating typical strains of oral streptococci [155], this method has been described as being able to successfully differentiate vital and avital bacteria in oral biofilms by Netuschil's group [184, 185]. The combination of SYTO 9 with PI seems to be appropriate for oral streptococcal strains [155, 186]. This combination has been successfully used for staining of PL-biofilm samples in numerous experiments from different research groups [85, 98, 114, 116, 171-174] and also by the author's group [88, 95, 96, 131, 134, 177, 178, 187, 188].

- SYTO 9/PI

Stains SYTO 9 and PI are found within the Live/Dead® BacLight™ Bacterial Viability Kit, developed by Molecular Probes Inc. (Leiden, The Netherlands) [175]. This combination has been widely used in the study of oral bacteria and biofilms, both *in vitro* and *in situ*, for the last 17 years [61, 85, 88, 95, 96, 98, 114-116, 129, 134, 171-178, 188].

The stain package is composed of a mixture of the two nucleic-acid-binding stains SYTO 9 and PI. These stains differ both in their spectral characteristics and in their ability to penetrate vital bacterial cells. SYTO 9 stains all cells green (vital and non-vital), whereas PI penetrates cells in which the membrane has been compromised or damaged, staining them red and stopping the emission of green fluorescence by SYTO 9 in these cells (Figure I.11) [189]. BacLight™ staining has several advantages; the

intensity of the fluorescence is high, with a strong contrast between the red and green cells, which makes the preparations easy to read with minimal background fluorescence. Furthermore, it is a reliable, rapid and easy-to-use test that yields both vital counts and total counts in one step [175].

The detection of vital cells using BacLight™ is based on the difference between the total cells and dead cells. SYTO 9 fluorescence is sensitive to the pH value, with the maximum fluorescence at a low pH (5.5 to 6.5) (Molecular Probes communication). As SYTO 9 penetrates all cell membranes, its efficiency can be limited by decreased membrane permeability to this stain or by the insufficient accumulation of the stain, so that does not become detectable. These same limitations can be applied to PI.

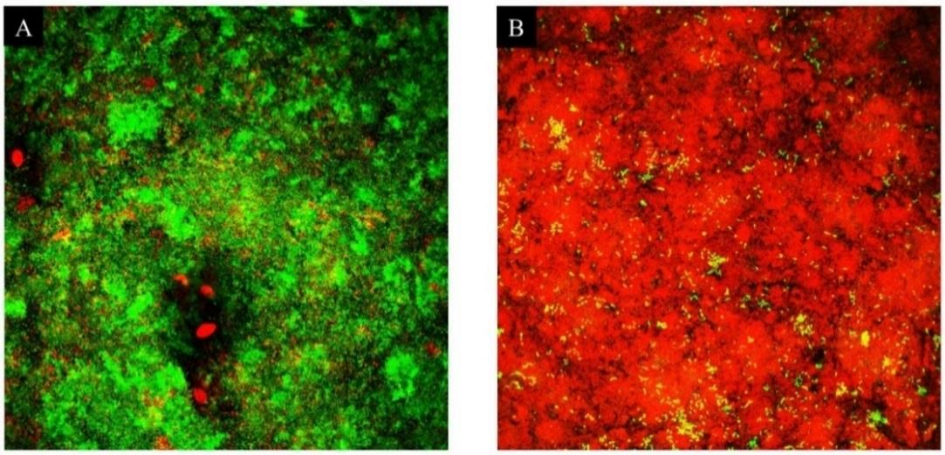


Figure I.11. Images of a PL-biofilm obtained with CLSM after staining with SYTO 9/PI. Vital bacteria are stained by SYTO 9 in green, and dead bacteria are stained by PI in red. A) The image in which the bacteria predominantly have intact membranes. B) The image in which bacteria predominantly have damaged membranes.

Depletion of cellular reserves, low cellular-protein content and degradation of macromolecules such as RNA and DNA will occur in starved cells, decreasing their concentration [190-192]. Theoretically, as SYTO 9

and PI are nucleic-acid-binding stains, it could be supported that, in starved cells, smaller amounts of nucleic acids may influence the ability to accumulate sufficient amounts of the stains to detect fluorescence. However, Boulos et al. [175] showed that, by comparing with other techniques, this hypothesis was wrong and the staining was correctly produced.

I.6. STRUCTURAL CHARACTERISTICS OF THE ORAL BIOFILM

In the literature, the importance of the structure-behaviour relationship of undisturbed PL-biofilm *in situ* and its implication in the pathogenesis of some oral infections has been recognised [193]. Specifically, Wood et al. [91] stated that the PL-biofilm structure probably represents an important factor in the modulation of bacterial physiology and the differentiation of the ecological niche. In this sense, the microbial behaviour will depend on parameters such as biofilm architecture, cell-to-cell relationships, thickness, bacterial viability (BV) and density –covering grade- [91]. As a result, a better knowledge of these structural features may favour the development of more effective strategies for the management of caries and periodontal diseases [72, 91, 93].

I.6.1. BIOFILM ARCHITECTURE

Biofilms have a definite architectural structure. The bacteria are not uniformly distributed throughout the biofilm; rather, there are aggregates of microcolonies that vary in shape and size. Channels between the colonies allow the circulation of nutrients and by-products and provide a system to eliminate wastes [91, 194].

Microorganisms on the outer surface of biofilms are not as strongly attached to the matrix and tend to grow faster than those bacteria deeper within the biofilm. Surface microorganisms are more susceptible to detachment, a characteristic that facilitates travel to form new biofilm colonies on nearby oral structures and tissues [35].

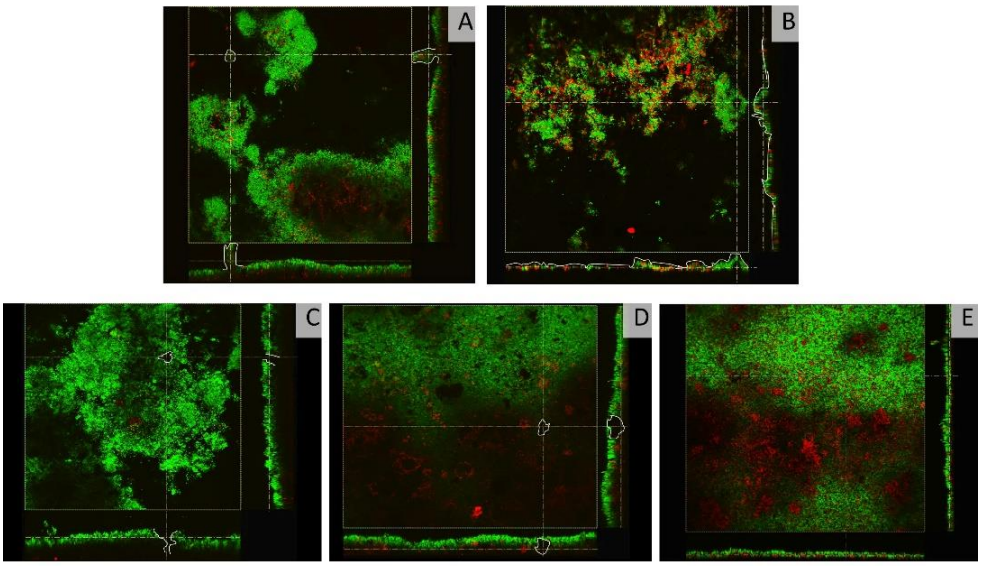


Figure I.12. Biofilm structure images acquired by CLSM. A) Mushroom shape; B) Irregular; C) Channels; D) Voids; E) Compact. The image was taken from Prada López et al. 2015 [96].

Based on the observations made by CLSM, many authors agree that the PL-biofilm has an open and heterogeneous architecture. This architecture is characterised by the presence of a complex system of channels and cavities or voids, which are part of the structure of the biofilm [69, 91, 93, 123, 170] (Figure I.12). These are not regularly distributed, since depending on the biofilm maturation grade. More voids and channels are found in the more mature biofilms [96, 106]. Although their functions have not been completely clarified [127], these channels and voids, presumably provide direct communication between the environment of the oral cavity

and the enamel surface [91]. This particular "circulatory system" could have important implications for moving the organic acids that damage the enamel, bacterial toxins and other antigens and for the release of antimicrobial agents on selected targets within the biofilm [91].

I.6.2. CELL-TO-CELL RELATIONSHIP

Bacteria in biofilm communicate with each other by a process called quorum sensing (QS). This dynamic, sophisticated communication system enables bacteria to monitor each other's presence and to modulate their gene expression in response to the number of bacteria in a given area of the biofilm [195]. In addition, as a result of QS, portions of the biofilm can become detached to maintain a cell density compatible with continued survival.

I.6.3. BIOFILM THICKNESS

In the literature, there are a good number of studies assessing the PL-biofilm, but only a few of them analysed its thickness. The first time that the thickness of the PL-biofilm was measured using the CLSM was in 1998. Netuschil et al. [109] observed that the PL-biofilm formed on enamel and glass disks depended not only on the “age“ of the biofilm but also, and mainly, from one individual to another; they found variations of more than 30 µm between individuals (from 6 µm to 45 µm after 3 days). This affirmation was posteriorly confirmed by other researchers [96, 134], obtaining a narrower range (from 10 µm to 33 µm for a 2-day PL-biofilm). In the same way, the findings found in other papers also corroborated the high variability but with even higher ranges (from 24 µm to 120 µm) and with a mean value of 76.7 µm (which doubles other obtained results in a 2-day PL-biofilm). In most cases, the thickness of a 2-day PL-biofilm has normally

ranged from 14 µm to 37 µm [72, 95, 127, 129, 134, 170, 174], although other studies obtained much higher thicknesses reaching even 150 µm [69, 70, 93]. These are noticeable methodological differences that will be further discussed in the corresponding section. Auschill et al. [123] stated that "*the height of the biofilms formed will depend on the plaque-forming rate of the individual donors*". Nevertheless, Zaura-Arite et al. [170] found no difference in the thickness of a 2-day PL-biofilm among heavy and light-plaque-formers (based on the average protein accumulation in samples of young plaque from smooth surfaces), although these authors used a grooved pattern model to ease the biofilm growing.

Although it has been observed that microbial deposits do not have a uniform thickness [127], an experiment compared the thickness of the PL-biofilm formed on glass disks after 2 and 4 days. The thickness values of those two periods were very close in this case (21 µm *vs.* 23 µm). Another study that assessed the increase in thickness over time was that from Al-Ahmad et al. [72], who observed that the biofilm suffered a process of maturation from 15 µm on the first day to almost 50 µm at 7 days. However, this increase was not regular and as previously stated, was individual-dependent [109]. Other investigations found that the thickness of oral biofilm at 3 days ranged from 7 µm to 34 µm [72, 109] and at 5 days from 15 µm to 45 µm [72, 123].

I.6.4. BIOFILM BACTERIAL VIABILITY

By the end of the twentieth century, some studies using conventional culture techniques and vital fluorescence, showed that in young dental plaque the dead material was predominant over alive microorganisms [196-198]. Netuschil et al. [109] found that living bacteria were found

preferentially in the higher part of dense strata containing dead material. Conversely, Zaura-Arite et al. [170] observed that living bacteria were mixed with dead material. This finding supports the hypothesis that dead bacteria, rather than living bacteria, adhere to solid surfaces, although another possible explanation lies in the antibacterial factors present in human saliva and their effects on PL-biofilm [186, 199-201].

The living microorganisms, located on these dead strata or embedded in them, may be responsible for the subsequent growth of plaque [109]. Dead bacteria provide nutritive supplements that favour the rapid expansion of living flora [109, 202] and protect it against antibacterial agents in the oral ecosystem itself [123]. Consequently, it has been determined that dead cell material represents a primary biomass component during the initial stages of accumulation and development of the PL-biofilm [109, 123]. In these biofilms, cavitated structures ("black holes") surrounded by live bacteria have been described, which could mean that these bacteria have direct access to the nutrients that diffuse through pores and cavities [203-205].

In the *in situ* studies by Arweiler et al. [93], Auschill et al. [70] and von Ohle et al. [107] on biofilm maturation after 2 and 3 days, an average BV of 60%-77% was reached. In another series, the viability in a 5-day PL-biofilm was 57%-63% [121]. Some authors have pointed out that BV increases as the biofilm maturation period and consequently its thickness [123, 170].

Large inter-individual differences in the disposition of live and dead bacteria have been described [170], so there does not appear to be a general pattern of BV distribution [107, 170]. In contrast, Arweiler et al. [93] suggested that in each participant in these studies there is a relatively constant ecological environment, which obviously leads to a pattern of

microbial identity. In this sense, Arweiler et al. [93] identified three patterns of viability in the PL-biofilm of 2 days. In the first pattern, a large number of dead bacteria (low viability) accumulate in the layers closest to substrate, viability increases in the higher strata and ends again with low values on the more superficial level of the biofilm. In the second pattern, when these bacteria are coated with living cells, or proliferate some of the bacteria that still survive or are cultivable, form a new layer of viable PL-biofilm. In the third pattern, the biofilm exhibits high values of BV in the layer which is nearest to the substrate, progressively decreasing towards the outside.

Auschill et al. [123] observed that the distribution of BV in PL-biofilm accumulated on smooth surfaces for 5 days had a similar profile to the first described pattern. The percentage of live bacteria was lower in the adjacent area to the surface of the enamel, increased following the z-axis towards the central zone and decreased again towards the outer layers. On the other hand, Arweiler et al. [121] demonstrated a pattern of viability more similar to the second one described in a 5-day PL-biofilm.They observed lower values in the lower strata (viability = 51%) and higher in the superficial layers (viability = 65%), confirming that bacteria close to the substrate are usually metabolically inactive (latent areas) [109, 206]. This last pattern was similar to what Prada-López et al. found in their study analysing a PL-biofilm of 2 and 4 days [96]. They detected that the BV was lower in deeper layers than in the higher ones. This finding coincides with the theory that the bacteria located in the deepest part of a biofilm are in an inactive metabolic state [91, 109]. Another interesting finding of this study was that the BV decreased as the biofilm matured mainly in the deepest layer. The possible reason for this decrease could be the higher thickness and density of the biofilm as the process of maturation advances [96].

I.6.5. BIOFILM COVERING GRADE

In the previous paragraph, the term density of the biofilm was introduced. This is a term that is directly related to the covering grade of the PL-biofilm [96] since this is defined as the percentage of the area of the substrate covered by the bacterial biomass. The more density a biofilm has the more area of the substrate it will cover. The covering grade is very useful when the antiplaque effect of an antiseptic agent is measured since it can be a predictive factor of its antiplaque activity. This is a concept that has been slightly forgotten in the available literature since very few studies evaluated this parameter [91, 96, 106]. It has been described that the covering grade of the PL-biofilm at 2 days is around 50%-60% [91, 96]. Wood et al. [106], found that the biofilm got denser as it matured, mainly in the layer that was nearer the substrate. Thus, a 4-day PL-biofilm reaches levels around 70%-80% of covering grade [96, 161].

I.7. ELIMINATION/CONTROL OF THE BIOFILM BY ANTIMICROBIAL AGENTS

The accumulation of bacterial biofilms on tooth surfaces results in two of the most prevalent infectious diseases of man –caries and periodontal diseases. Although prevention and control of these diseases may be achieved by daily mechanical removal of the biofilms, many individuals are either unable or unwilling to practice these procedures as regularly or as efficiently as is necessary. There is, therefore, great interest in the possibility of using chemicals to replace or augment mechanical preventive and therapeutic procedures [57, 206, 207].

The extracellular matrix produced by biofilm bacteria encloses the microbial community and protects it from the surrounding environment,

including attacks from chemotherapeutic agents. Chemotherapeutic agents have difficulty penetrating the polysaccharide matrix to reach and affect the microorganisms [4, 15, 208, 209]. Thus, the matrix helps to protect bacteria deep within the biofilm from antibiotics and antiseptics, increasing the survival of the colonies. Furthermore, the extracellular matrix keeps the bacteria banded together, so they are not flushed away by the action of saliva and gingival crevicular fluid.

The methods to control the development of dental biofilms are traditionally divided into two: physical and chemical elimination (Figure I.13). In the last decade, a new component has been added to this equation, the probiotics and prebiotics [210]. Probiotics are biologic components which can successfully manipulate the microbial composition in the oral cavity to improve the clinical conditions such as halitosis [211], candidiasis [212], tooth decay [213, 214] and periodontal diseases [215]. Prebiotics do not follow the manipulation of the microbial composition in the oral cavity with external biologic compounds; they are substrates that are selectively utilised by microorganisms conferring a health benefit [216]. In any case, this branch of the scheme needs further research to be a useful method for the control of the oral biofilm development and its pathogenicity.

The physical methods may be done professionally or at home and are based on the scaling and root planning and the oral hygiene done at home with the toothbrush, the dental floss, interdental brushes or oral irrigation devices. The chemical methods include the antibiotics and the antiseptics. This Thesis will be focused on the action of the antiseptics in the oral biofilm.

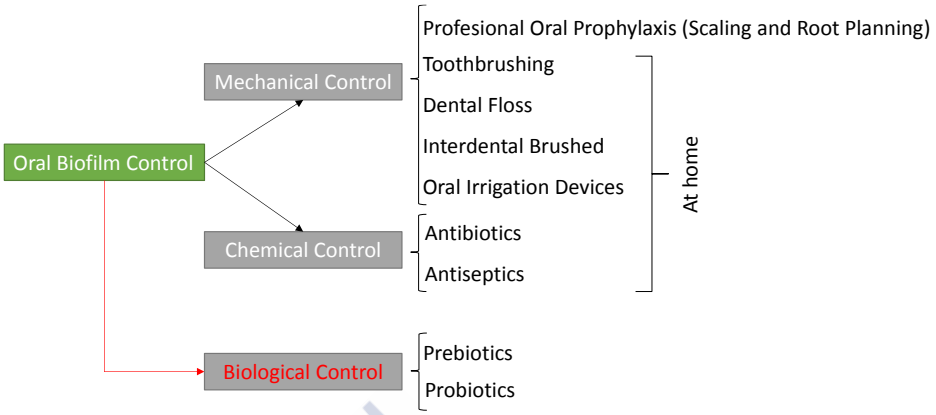


Figure I.13. Scheme of strategies to control oral biofilm.

The active principles present in the mouthwashes which are most commonly used in the oral cavity include chlorhexidine (CHX), combinations of essential oils (EO), triclosan, cetylpyridinium chloride, and various metal salts such as zinc compounds and stannous fluoride. Of all of them, CHX mouthwashes are considered the *Gold Standard*, since they have so far been the most effective in microbiological and clinical studies [107, 217, 218].

A prerequisite for an antimicrobial agent to be effective is that all the bacteria integrating the biofilm are exposed to an adequate concentration of that named agent for a given time so that the biofilm is reduced in a clinically objective and quantifiable manner [107]. The penetration rate of the antimicrobial agent, as well as the depth it reaches in the thickness of the biofilm, depend on factors such as the structure and composition of the biofilm [71, 91], although its thickness [219] and physicochemical characteristics [71, 91] are probably more important.

It has been found that the transport of particles in the biofilm is not an instant process [71]. The passage of solutions with high concentrations of

solutes through thick biofilms (>1 mm) requires continuous and prolonged exposure, and that penetration is further delayed if the solute interacts with biomass [220].

Although it has not been definitively confirmed if the PL-biofilm architecture influences the rate or pattern of particle transport, it has been shown that the transfer of solutes through the channels of a non-dental biofilm is faster than through dense biomass. This is because the resistance to diffusion is less [220] and because of the convection phenomenon [205]. While convection may be minimal in the PL-biofilm, there is some evidence that the rates of solute penetration through biofilm are inversely proportional to the biomass density [221]. The density of PL-biofilm increases from the outer layer to the inside [96, 111], which explains why the solutes diffuse more easily in the outer layers of the plaque than among the deeper ones [71]. However, in a 7-day PL-biofilm model, Robinson et al. [111] found that, at least, its outer layers were resistant to mechanical and chemical treatments (exposing biofilm to sodium chloride at 0.9% and 9%, as well as to an acid pH), but this scenario changed dramatically in the presence of detergents.

I.7.1. GENERAL CHARACTERISTICS OF THE ORAL ANTISEPTICS

The ideal properties of an oral mouthwash include: being fast and safe, capable of removing bacteria from the biofilm in areas of difficult access, good taste, economical and easy to use [222]. To be considered an antiseptic, it must be a product applied to living tissues that destroys or inhibits the proliferation of pathogenic microorganisms without injuring host cells. The present Thesis will be focused on the most commonly prescribed antiseptics in dentistry: the EO and the CHX.

I.7.1.1. ESSENTIAL OILS

- History of the intraoral application of the essential oils

Since ancient times, the EO have been used for the treatment of a large variety of diseases all over the world, from the Egyptians to the Mayas or Aztecs [223]. In 1879, the Dr. Josep Lawrence and the pharmaceutic Jordan Wheat Lambert developed a phenolic compound [224], whose antimicrobial activity was enhanced by the combination with some EO (Thyme, Eucalyptus, Baptisia, Gahulteria and Mentha Arvensis) [225]. This formula was called Listerine® in honour of Sir Joseph Lister, father of the antisepsis in medicine [226]. Although it had been designed as an antiseptic for surgeries and it also had demonstrated its antimicrobial activity, Listerine® had a poor acceptation among the surgical medicine field. However, Listerine® soon acquired other indications such as treatment of gonorrhoea, floor cleaner, anti-dandruff solution, aftershave lotion or remedy for baldness (Figure I.14). After that, it was observed that Listerine® was especially effective against germs commonly found in the oral cavity. As a result, in 1895, Lambert extended the sale and promotion of his product to the dental profession [224]. It was his son, Gerald, who introduced the product to Americans as a palliative for halitosis (Figure I.14), becoming so popular that in 1914 was one of the first prescription oral products and technically reached the category of mouthwash. By this time, the oils included in the original formula had been already replaced by eucalyptol, thymol, menthol and methyl-salicylate -the latter two are replaced by synthetic derivates today- [227]. In the decades of 70s-80s, Listerine® started to be considered as a mouthwash against not only the halitosis but also other mouth diseases [228]. This condition became true in 1986 when the American Dental Association approved them for the

control of the dental plaque and gingivitis [229] based on some existing studies that satisfied their quality criteria [230-233].

Figure I.14. Advertisements at the beginning of the XX century in which the different uses of Listerine® were announced.

Nowadays, Listerine® is widely used all over the world and has been used by millions of customers, particularly in the United States [234]. Furthermore, it has been considered as efficient and safe by the Expert Committee on Oral Health of the Food and Drug Administration (FDA) [235].

- General characteristics of the essential oils

- Origin and obtaining

The EO are produced by the secondary metabolism of aromatic and medicinal plants. They can be achieved in different ways, including the use of low or high pressure distillation of various parts of plants, the use of liquid carbon dioxide or microwave application [223, 236].

- Chemical structure

The EO are composed of a complex mixture of volatile molecules, within which two groups can be differentiated. The main group is derived from a simple terpene or one containing oxygen (terpenoid) and the second is constituted by an aromatic component (Figure I.15). Terpenes are the largest group of products extracted from natural plants characterised by a wide variety of structural types [237]. The monoterpenes (composed of10 atoms of carbon -c10- Figures I.16 and I.17), sesquiterpenes (c15) and diterpenes (c20) are the major terpenes, but hemiterpenes (c5), triterpenes (c30) and tetraterpenes (c40) may be also found. The other group based on an aromatic component, which is less common than terpenes, are derived from phenylpropane (Figures I.18 and I.19).

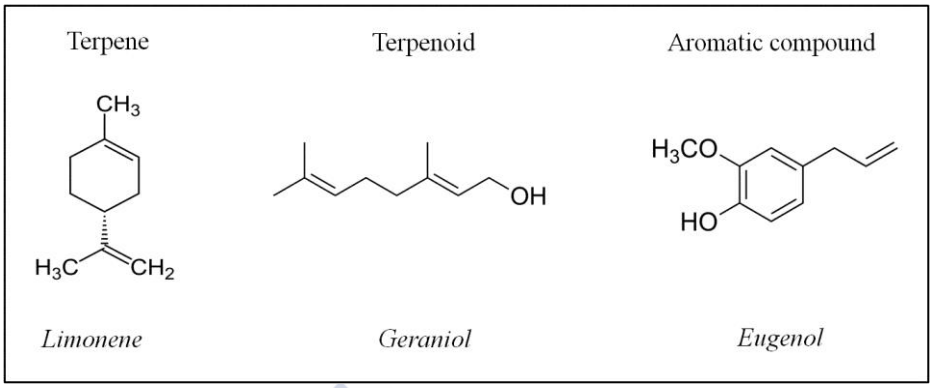
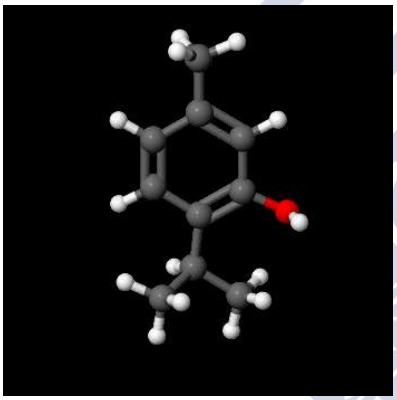
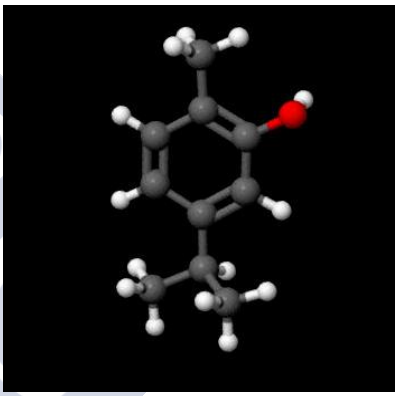
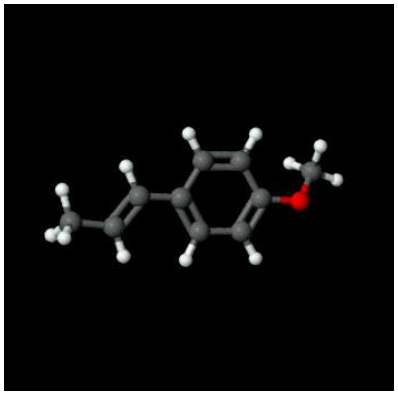


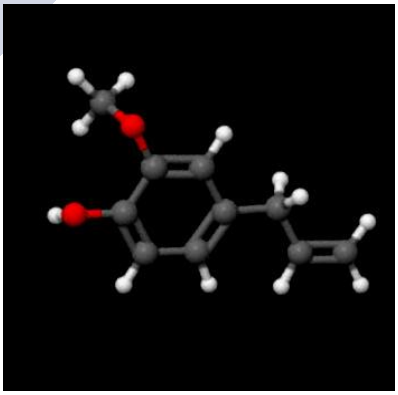
Figure I.15. Chemical formulas, which are exemplifying the different types of EO.





Figures I.16. and **I.17.** Chemical structure of monotherpenic EO: thymol and carvacrol.





Figures I.18. and **I.19.** Chemical structure of the aromatic EO: anethole and eugenol.

- Physical properties

The EO are volatile and water-insoluble which makes very difficult the determination of their antibacterial effect [238]. Moreover, their high hydrophobicity and viscosity cause an irregular distribution when testing their antiseptic activity. Due to this, it is always necessary a solvent agent to avoid the unequal dilution in the culture medium [239]. Other factors considered key in the evaluation of their activity are: the composition of the culture medium, the microbial species studied, the pH and the temperature, as well as the microbiological technique used [240-242].

- Mechanism of action

The EO have a complex mechanism of antibacterial action [243], in fact, it was unknown until 1985 [232]. Even so, the antimicrobial effects of the EO are intimately attached to their dominant characteristic, the hydrophobicity, which produces an increase in the bacterial membrane permeability and the consequent loss of their primary cellular elements [244-247]. It is important to remark that a perturbation in the bacterial covers (membrane and wall) can affect to other cellular compounds and produce a cascade of events [248] (Figures I.20 and I.21). In the following paragraphs, the different actions that EO provide on the bacterial cell are explained in more detail.

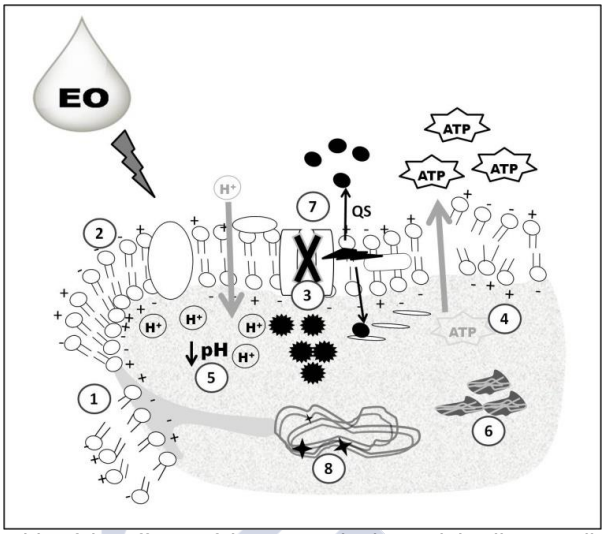


Figure I.20. Graphic of the effects of the EO on the bacterial cells. 1. Cell wall and membrane disturbance. The increase in the cell permeability. 2. Loss of the cell membrane potential. 3. Disturbed trans-membrane transport, accumulation of toxins inside the cell. 4. Intracellular ATP decrease. The ATPs go out of the cell due to the membrane-increased permeability. 5. Intracellular pH falls because of the inability of the membrane to block the extracellular protons. 6. The appearance of coagulated material in the intracytoplasmatic cell space. 7. Inhibition of the "Quorum Sensing". Stopping of the intra- and intercellular communications. 8. The appearance of mutations in the DNA.

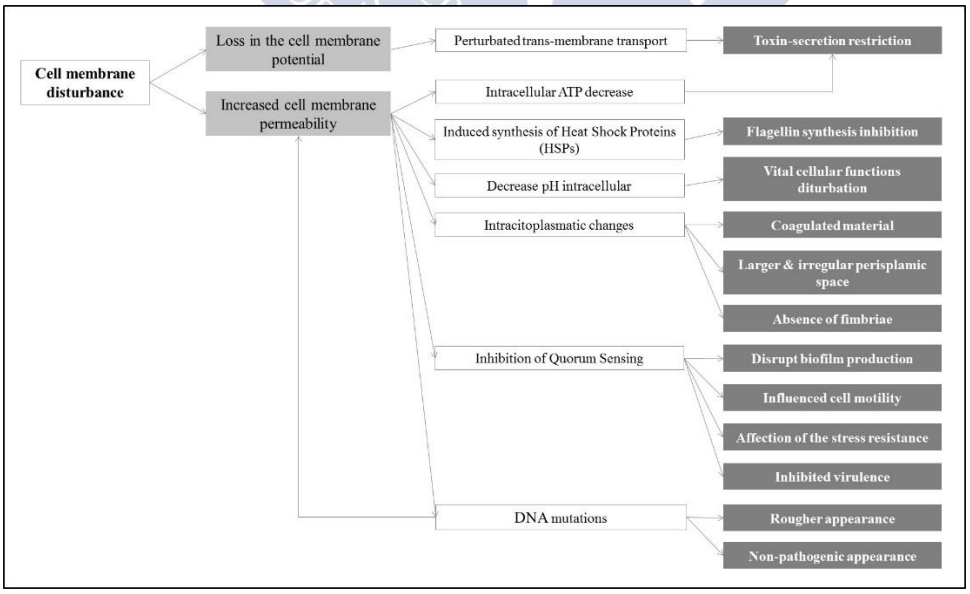


Figure I.21. Scheme of the mechanisms of antibacterial action of the EO.

Membrane and cell wall disturbance

The evaluation of the loss of cellular constituents, such as lipopolysaccharides, helps to establish the severity of cell membrane damage. This alteration has been analysed by some authors to clarify the antibacterial action of EO [248-250].

The EO cause a loss of membrane potential, which severely affects the transport mechanisms [250]. There are differences of affection between Gram-negative and Gram-positive bacteria (mainly due to the different composition of the membrane and the bacterial wall), resulting in a greater action in the Gram-negative bacteria [250].

The EO produce the appearance of "white spots" on the cell wall of Gram-negative bacteria [251] and imperfect cellular structure [246]; while in Gram-positive bacteria, only an abnormal cell form is observed [252]. This abnormal cellular structure implies surface malformation and the development of invaginations of the cytoplasmic membrane, with cytoplasmic content aggregation [253, 254] and induction of the formation of a very rough and thickened wall [255].

Consequently, there is an indirect inhibitory action on the production of toxins [256, 257], because structural changes caused by the binding of EO that disrupt the lipid bilayer, condition the trans-membrane transport, thereby limiting the release of toxins into the medium [249].

ATP production

The destructuring of the cell membrane by any antimicrobial agent, including EO, will compromise some vital functions such as nutrient processing, synthesis of structural macromolecules and secretion of the

major growth enzymes. The production of ATP in prokaryotes occurs both in the cell membrane and in the cytosol by the glycocalyx. Therefore, the balance of intra- and extracellular ATP is expected to be altered by the EO action on the cell membrane. In fact, a negative correlation has been found between intra- and extracellular ATP concentration [246, 252, 258], resulting in a high loss of ATP through the membrane altered by the EO [246, 252].

Protein synthesis

Burt et al. [237] first described the action of EO on protein synthesis. The components of the EO, carvacrol and cymene, induced the synthesis of HSPs ("Heat Shock Proteins"). HSPs are molecular chaperones that are related to different assembly and release processes of polypeptides, whose synthesis is enhanced when bacteria contact toxic substances or other stress conditions. In the study by Burt et al. [237], *Escherichia coli* O157: H7 bacteria, incubated overnight in the presence of 1mM carvacrol, produced significant amounts of HSP60 and inhibited flagellin synthesis, resulting in non-motile bacteria.

Intracellular pH perturbation

The maintenance of intracellular pH (pH_{in}) at appropriate levels is critical for achieving fundamental cellular processes (DNA transcription, protein synthesis and enzymatic activity) when the cell is exposed to severe damage [249-251].

The pH_{in} in bacterial cells exposed to EO has been evaluated, detecting a significant reduction [246, 252]. The homeostasis of the pH can be affected by the action of the EO on the membrane, which loses its capacity

to block protons [245, 246, 252]. In the study by Turgis et al. [246], a significant decrease in pH_{in} was observed, since it changed from 6.23 to 5.20 for *E. coli* 0157: H7 and from 6.59 to 5.44 for *Staphylococcus typhi,* when bacteria were treated with the minimum inhibitory concentration (MIC) of mustard EO.

Intracytoplasmic changes

In the paper conducted by Becerril et al. [259], *E. coli* cells treated with the EO of oregano exhibited intracytoplasmic changes, such as coagulated material in particular areas located in the cell wall and at the apical boundaries. When *E. coli* bacteria were treated with cinnamon EO, the periplasmic space showed significant changes, in particular, they became larger and more irregular. The researchers also noted the absence of fimbriae in the altered periplasmic space. *Staphylococcus aureus* bacteria, treated with both oregano and cinnamon EO, exhibited the same cellular malformations as *E. coli*, but in a less pronounced manner.

DNA disturbations

Since bacterial DNA is physically bound to the cell membrane, it is expected that the EO will also act on the DNA. This fact has been used to measure the genotoxicity and mutagenic effects of EO and other agents [253-256, 260]. There are several tests to quantify these effects [253, 254] based on the detection of different mutations, produced in bacterial chromosomes, which affect the polysaccharide side chain of the lipopolysaccharides that cover the bacterial surface. These mutations confer an appearance of pronounced roughness to the bacterial cells, being highly permeable and completely harmless.

Quorum Sensing

Bacteria produce signalling molecules used in cell-to-cell communication QS to evaluate their external environment and their internal physiological status, thus helping to modulate their populations [257]. In general, these molecules are known by/as autoinducers. Gram-negative bacteria use acyl-homoserine lactones, while Gram-positive bacteria use modified oligopeptides [261].

QS is involved in the production of biofilm, motility, resistance to stress and virulence [262]. The important role attributed to QS in bacterial life makes this process an attractive target for controlling infections and decreasing antimicrobial resistance [263]. Research on the anti-QS activity of EO or their components is in continuous progress [264-266]. Recently, it has been shown that EO such as those extracted from rose, geranium, lavender or clove seem to be very effective in inhibiting QS [265, 267].

- Chemical composition of the Listerine® Mentol™ and Listerine® Zero™

The EO are composed by a wide diversity of products. Therefore, their antimicrobial activity will be related to their composition, configuration, amount and their possible interactions [268]. Three effects can be highlighted: additive, antagonist and synergetic. The additive effect occurs when the combined effect of the components is equal to the sum of the individual effects. Synergism is registered when the activity of the combined substances is higher than the sum of the individual activities. In contrast, the antagonistic effect is present when the activity of components in combination is inferior in comparison when they are applied separately.

The two formulas containing EO used in the present Thesis are Listerine® Mentol™ (Listerine®, Johnson & Johnson, Madrid) and Listerine® Zero™ (Listerine®, Johnson & Johnson, Madrid). Both of them are a complex mixture of phenolic compounds combined with several EO. The composition of Listerine® Mentol™ is: 0.092% of eucalyptol, 0.064% of thymol, 0.06% of methyl-salicylate and 0.042% of menthol. All of this solved in a hydroalcoholic vehicle containing a percentage of 21.6% to 26.9% of alcohol [269].

- Eucalyptol (Figure I.22)

It is a volatile oil extracted by steam distillation of fresh leaves of *Eucalyptus globulus*. Eucalyptol is colourless, somewhat camphorated, with a spicy, refreshing taste. It is also known as cineol. It is insoluble in water but miscible with alcohol, chloroform and ether. It is employed as an anaesthetic/analgesic active agent for topical use in the oral and pharyngeal mucosa. It can be used in mouthwashes when used in concentrations of 0.025% to 0.1% [270].

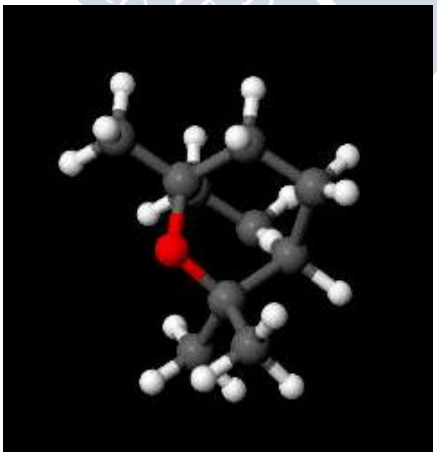


Figure I.22. Chemical structure of the eucalyptol.

- Thymol (Figure I.23)

It can be prepared synthetically or obtained by distillation of volatile oils from *Thymus vulgaris* and other related plant sources. It is an alkyl derivative of phenol that has bactericidal and fungicidal properties [270].

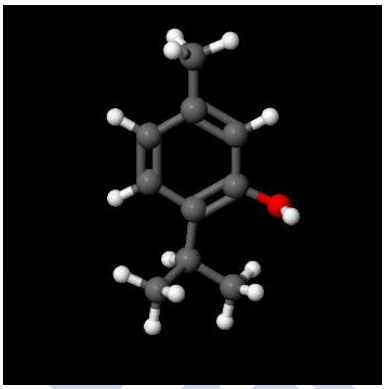


Figure I.23. Chemical structure of the thymol.

- Menthol (Figure I.24)

It is a secondary alcohol that can be extracted from peppermint oil or produced synthetically by the hydrogenation (reduction) of thymol. It can be used topically in the oral cavity as a mouthwash as active ingredient at concentrations ranging from 0.04% to 2% [270].

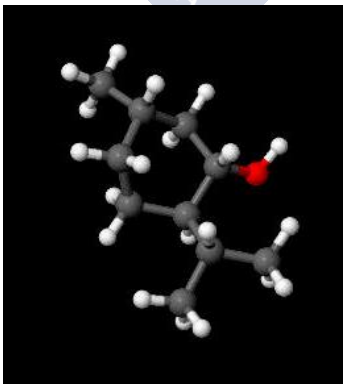


Figure I.24. Chemical structure of the menthol.

- Methyl-salicylate (Figure I.25)

It is a methyl-ester of salicylic acid. Before the discovery of the method for the chemical synthesis of methyl-salicylate, it was extracted from natural sources such as wintergreen, birch or betula. Today, it is prepared synthetically by the esterification of salicylic acid with methanol. It is accepted for topical use in mouth and pharynx mouthwashes in concentrations not higher than 0.4%, and no more than 3 or 4 times daily [270].

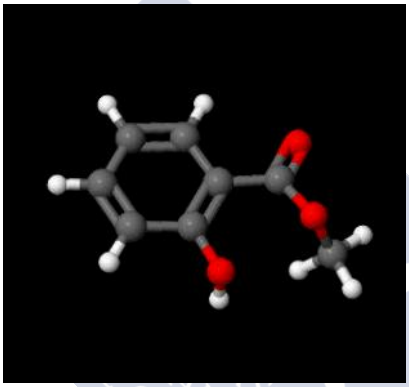


Figure I.25. Chemical structure of the methyl-salicylate.

The composition of the Listerine® Zero™ (Listerine®, Johnson & Johnson, Madrid) is the same in their active ingredients (eucalyptol, thymol, methyl-salicylate and menthol), but sodium fluoride has been added. Some differences are found in their inactive ingredients. These are based on the alcohol containing the Mentol™, without a presence in the Zero™ and the presence of propylene glycol, sodium lauryl sulphate and sucralose in the Zero™, without a presence in the Mentol™ (Table I.4).

- *The alcohol content is a matter of controversy*

Traditional mouthwashes of EO contain ethanol, which is a chemical compound used to dissolve and stabilise the numerous substances present in the rinse. The concentration of ethanol present in the traditional EO mouthwashes, as previously said, is more than 20%. This level has been found sufficient to dissolve the EO but insufficient to have a direct antibacterial effect [271, 272]. In fact, the manufacturer presents the alcohol content (21.6%), among others, as an inactive ingredient in its formula (Table I.4) [273]. Over the years, the adequacy of the use of ethanol in mouthwashes, as well as their effects on the surfaces of composite restorations [274] and their possible role in the development of oropharyngeal cancer have been discussed [275, 276].

Table. I.4. Ingredients present in Listerine® Mentol™ and Listerine® Zero™.

ACTIVE INGREDIENTS					
	Eucalyptol	Thymol	Methyl-Salicylate	Menthol	Sodium Fluoride
Listerine® Mentol™	V	V	V	V	X
Listerine® Zero™	V	V	V	V	V

COINCIDENT INACTIVE INGREDIENTS							
	Benzoic Acid	Flavour	Poloxamer 407	Sodium Benzoate	Sodium Saccharin	Sorbitol	Water
Listerine® Mentol™	V	V	V	V	V	V	V
Listerine® Zero™	V	V	V	V	V	V	V

NON-COINCIDENT INACTIVE INGREDIENTS				
	Alcohol	Propylene Glycol	Sodium Lauryl Sulphate	Sucralose
Listerine® Mentol™	V	X	X	X
Listerine® Zero™	X	V	V	V

A direct cause-and-effect association between the development of oropharyngeal carcinoma and the use of alcohol-based rinses has not been demonstrated [277-279] and probably it will never be (at least by epidemiological studies) [280]. However, it is considered desirable to

eliminate ethanol from daily mouthwashes, especially in pediatric populations and patients at high risk of cancer [281, 282], leading to the development of new formulations.

I.7.1.2. CHLORHEXIDINE

- History of the intraoral application of the chlorhexidine

In 1947, a complex study to synthesise new antimalarial agents led to the development of the poly-biguanides [283]. These compounds showed a significant antimicrobial potential, particularly compound 10,040, a cationic detergent later called CHX [284]. Although two salts with similar antibacterial activity, the diacetate and the dihydrochloride, were initially selected, the diacetate was more soluble and was, therefore, more suitable for laboratory work. Davies et al. [284] demonstrated that this compound had bacteriostatic activity, especially against Gram-positive bacteria and bactericidal activity. Experimental studies in albino mice revealed a low degree of toxicity at 10 days after the subcutaneous, intraperitoneal, intravenous or oral administration of a single dose of CHX, as well as after a year of continuous oral administration.

The first salt derived from compound 10,040 that reached the market was CHX gluconate, which was registered in 1954 by Imperial Chemical Industries Co. Ltd of Macclesfield (United Kingdom) as Hibitane®, the first internationally accepted antiseptic for cleansing wounds and the skin [285, 286]. In 1957, only 3 years after coming onto the market, the broad antimicrobial spectrum of CHX led to an extension of its indications to include not only skin disinfection but also use in the fields of ophthalmology, urology, gynaecology and otorhinolaryngology. Although CHX started to be used to control bacterial plaque in 1959, it was not until the publication of

the studies by Löe and Schiött in the 1970s that the use of CHX became widespread in dentistry [43, 287].

Nowadays, the CHX is considered to be the oral antiseptic par excellence. The most commonly used concentrations in commercially available CHX mouthrinses are 0.2% and 0.12%. Besides its effects on plaque and gingivitis, CHX is effective in the prevention and treatment of caries, infections secondary to oral surgical procedures, and in the maintenance of implants.

One of the main advantages of CHX is its ability to bind to a wide variety of substrates while maintaining its antibacterial activity. It is then slowly released, leading to persistence of effective concentrations; this property is known as substantivity [288].

- *General characteristics of the chlorhexidine*

- Chemical structure

The CHX is an amphipathic molecule with hydrophilic and hydrophobic groups and is cationic at physiological pH. This molecule was synthesised from proguanil and belongs to the biguanide family, a group of compounds with antimalarial activity [289]. Structurally, it consists of two symmetrical chlorophenol rings (4-chlorophenyl) and two biguanide groups, united by a central hydrophobic hexamethylene chain. The result, as shown in Figure I.26, is a symmetrical bicationic molecule called 1,6-di(4'-chlorophenyl-diguanide)hexane [290].

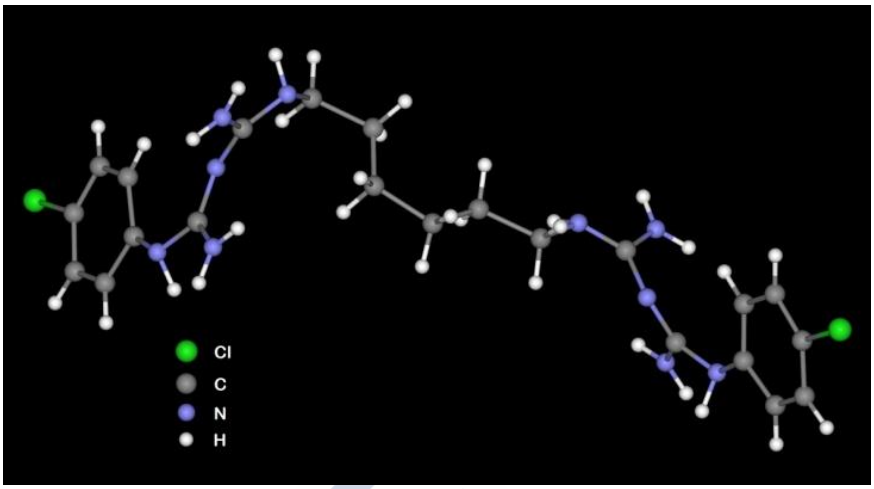


Figure I.26. Chemical structure of chlorhexidine (C22H30Cl2N10).

Usually, CHX is used in salt form, principally as diacetate, digluconate or dihydrochloride, as the base molecule is relatively insoluble in water [285, 291]. Digluconate salt is the most soluble in water and alcohols and is, therefore, the form employed in the majority of mouthrinses, gels and varnishes. Also, this CHX salt has the added advantage that, at physiological pH, it releases the active component with a positive ionic charge [292].

- Physical properties

The CHX is a strong dicationic base, stable at a pH between 5 and 8 [291], although the optimum is between 5.5 and 7 [292, 293]. Consequently, the pH of the oral medium significantly affects the binding of CHX to dental and gingival surfaces, as well as the subsequent release of active CHX [294]. It has also been shown that its permanence in the mouth is much lower when the pH of the mouthwash used is very acidic (1.5-3), compared to that of neutral or alkaline solutions [295]. This observation is probably

because with the acidification reduces the number of negatively charged receptors; however, pH increase does not lead to a proportional increase in CHX retention [294]. Moreover, its activity against Gram-positive, Gram-negative bacteria and yeasts is higher when the pH is in the range of 5-8 [292, 293].

The CHX is photolytic, so it must be protected from exposure to light. Although stable at room temperature, it is also thermolabile and decomposes with heat to chloroaniline (a highly polluting compound with mutagenic properties); prolonged storage favours the production of anilines. European sanitary authorities have set maximum permitted values for chloroaniline in CHX preparations for human use at 500 ppm, and most of the solutions available on the market are only around 5 ppm and therefore have a wide margin of safety. Experimentally, after storing CHX samples for 6 months at 40°C, the limiting chloroaniline concentration was not achieved [285]. In any case, it is recommended to keep this antiseptic in a cold and dark environment and replenish it with some frequency.

It has been suggested that heat increases the antibacterial activity of CHX. König et al. [83] demonstrated that hot 0.2% CHX solutions provided greater reductions in supragingival plaque viability than cold solutions (47 *vs.* 21%). These authors further noted that heating an aqueous solution of CHX gluconate at 60 °C did not produce p-chloroaniline, but instead, its storage a high temperature increased the level of impurities. These findings coincided with those previously obtained by other authors [296].

Although many researchers argue that the presence of organic matter readily inactivates CHX [297], this claim has given rise to some controversy. It has been suggested that some plasma proteins could be combined with

the dicationic CHX molecule through its outer negative charges by blocking it, so it is not recommended as an antiseptic of the first choice for disinfection of bloody wounds or lesions that occur with interruption of the integrity of the oral mucosa [285].

Some authors believe that in addition to blood, pus can also inactivate CHX [283], although this view is not shared by other researchers [292, 298]; it has also been noted that organic detritus generated by a scaling and root planning procedure does not modify the CHX activity [299].

The CHX forms soluble salts with nitrates, sulphates, carbonates and phosphates [291]. However, it is incompatible with anionic surfactants such as sodium lauryl sulphate, a surfactant that is incorporated as an excipient in many conventional dentifrices [285, 291]. Although the clinical efficacy of the association of CHX and fluoride for the prevention of caries and gingivitis has been documented in numerous studies [300-303], the mechanism of association of anionic molecules such as fluoride with cationics such as CHX remains controversial [286]. In this sense, it seems that the combination of both active principles has no synergistic effect regarding inhibition of *de novo* plaque formation nor in the reduction of salivary bacterial content [304]. On the contrary, it has also been shown that the incorporation of sodium fluoride reduces the substantivity of CHX [305].

- Mechanism of action

The concept of "target" used to explain the mechanism of action of antibiotics may also apply to CHX; however, unlike the antibiotics, it does not break down the bacterial wall [306]. CHX, like other cationic antimicrobials known for decades as "membrane-active agents" [290], acts

on the cell membrane to increase its permeability and facilitate the release of intracytoplasmic material.

The outer layer of the bacterial cell wall carries a negative charge, usually stabilised by the presence of cations such as Mg^{2+} and Ca^{2+}; for this reason, the majority of antiseptics are cationic agents that have a high affinity with the bacterial cell wall [290, 307]. The first phase of action of CHX is its adsorption onto the wall of the microorganism [292].

This phase is a very rapid process that is explained by the presence of two basic and symmetrical clorophenylguanide groups attached to a lipophilic chain of hexamethylene and which form the bicationic molecule that readily interacts with the bacterial surface [308]. Conditions for binding are favourable when the pH is neutral or slightly alkaline; the amount of CHX adsorbed is dependent on the concentration of the agent. Although the mechanism of action of the bisbiguanides is very similar to that of the quaternary ammonium compounds, their binding to phospholipids and proteins from the bacterial cell wall and membrane is stronger. In addition, the length of the hydrophobic region of the bisbiguanides prevents their interdigitation within the lipid bilayer. CHX thus establishes a bridge between pairs of adjacent phospholipids and displaces the associated divalent cations.

The cationic CHX molecule will bind principally to anionic compounds such as free sulphates, lipopolysaccharide phosphate groups and protein carboxyl groups [309]. At low concentrations, CHX increases the permeability of the cytoplasmic membrane, altering its metabolic osmoregulatory capacity and its enzyme content [289, 310]. Some indirect indicators of these effects are the escape of potassium ions, phosphorus

and protons, and an inhibition of respiration and solute transport. This alteration of membrane integrity is responsible for the bacteriostatic effect of CHX and is reversible [293, 298]. At higher concentrations of CHX (more than 0.1%), the interactions are stronger, with crystallisation of the membrane that causes loss of its structural integrity, leading to a catastrophic release of intracellular material. This action is the basis of the bactericidal effect of CHX, which causes precipitation or coagulation of the bacterial cytoplasm, with a paradoxical decrease in the outflow of components through the membrane. This process eventually leads to cell death [311, 312].

The CHX is a broad-spectrum antiseptic, bactericide and fungicide [298]. Although it is not considered to be virucidal, CHX shows some activity on the lipid envelopes of viruses such as HIV, Herpes 1 and 2 and Influenza A [291, 313]. The CHX is not sporicidal, nor does it eliminate acid-fast bacteria [292], though it can inhibit the growth of spores and has a bacteriostatic effect on some mycobacteria [291]. CHX is more effective against Gram-positive than against Gram-negative bacteria due to differences in the structural characteristics of the outer membrane [286].

- *Chemical composition of the Oraldine® Perio™*

In the market, there are plenty of mouthwashes containing CHX in their formula. Those which use the molecule alone at a concentration of 0.12% or 0.20%, those which use it in combination with cetylpiridinium chloride, zinc or sodium fluoride and those which added alcohol to the formula. Although all combinations have shown a good antibacterial activity, some differences have been found between them [218, 304, 314-318]. In the present Thesis, a CHX not associated with others compounds and

manufactured by the same laboratory producing the EO was chosen (Table I.5). This decision was adopted in order not to introduce biases not only in the active ingredients but also in the inactive ones, since they may interact somehow in the final antibacterial effect.

Table I.5. Active and inactive ingredients present in the Oraldine® Perio™ formulation.

ACTIVE INGREDIENTS	
0.2% Chlorhexidine	
INACTIVE INGREDIENTS	
Water	Poloxamer 407
Sorbitol	Aroma
Maltitol	Sodium Saccharin
Propylene glycol	Citric Acid

I.7.2. EFFICACY OF THE ESSENTIAL OILS AND CHLORHEXIDINE ON ORAL BIOFILM

To measure the effectiveness of mouthwashes against the dental plaque two different concepts should be defined: the substantivity and the antiplaque effect.

The substantivity of an oral antiseptic is defined as the prolonged adherence to the oral surfaces (teeth and mucosa) and its slow release at active doses which guarantee the persistence of the antimicrobial activity [288]. The more substantivity an oral antiseptic has, the better. For its study *in situ*, the most popular models are those which analyse the effect that a single mouthwash has in a mature biofilm (more than 24 hours) [89, 134, 177, 188].

The second aspect that should be studied from an oral antiseptic, the antiplaque effect, is defined as the capacity that an agent has to avoid the

formation of bacterial aggregates (plaque) on the oral surfaces. For its study *in situ*, models start from a baseline sample with levels of plaque near to assess the power of the antiseptic to reduce the formation of bacterial plaque (normally dental plaque) against the control. A 6-month clinical study using a determinate antiplaque agent is necessary to label an antiseptic as effective. However, in the literature, there is an established model of 4 days of plaque regrowth with which can assess the inhibitory activity that the mouthwashes have *per se.* Furthermore, it determines the relative efficacy of the different formulations being considered as predictable of the antiplaque effect of an antiseptic [81, 84, 120, 122, 319-325].

I.7.2.1. IMMEDIATE ANTIBACTERIAL EFFECT AND SUBSTANTIVITY OF THE ESSENTIAL OILS ON ORAL BIOFILM

Although *in vitro* studies do not necessarily have to be predictive of clinical activity, they may help elucidate the underlying mechanisms. Thus, we find different studies that aim to determine the *in vitro* antimicrobial activity of EO, using biofilm models that try to represent the biofilm that is formed on the surface of the teeth [6, 125, 162, 173, 326, 327].

On the other hand, we have so-called *in situ* studies, which pursue the same objective but have a greater weight in establishing the antiseptic efficacy of the various mouthwashes, since the activity is tested on actual clinical conditions [87, 89, 98, 174, 328].

- *In vitro activity*

Pan et al. [326] attempted to predict with an *in vitro* study that the rinsing of EO has higher antimicrobial activity on bacterial biofilm than that

containing amine fluoride, a fact that would subsequently be rediscovered using, a year later [87], an *in situ* study model.

In the same year, Fine et al. [329] studied the susceptibility of different types of *Streptococcus* spp. to the EO. They obtained a reduction in viability that ranged from 85.7% for *Streptococcus gordinii* to 99.7% for *Streptococcus sobrinus*. In this same study, they performed an *in situ* test on 29 volunteers, who rinsed for 11 days twice daily with 30 mL of Listerine® to check if there were significant reductions in a number of bacteria of the genus *Streptococcus*, both in plaque and in saliva. The reduction results obtained were 69.9% (75% for *Streptococcus mutans*) in PL-biofilm and 50.8% in saliva (39.2% for *S. mutans*).

The same research group [6] continued on the path of *in vitro* studies. They compared the antimicrobial activity of EO, with an amine fluoride rinse and a triclosan rinse, on a 3-day biofilm model course containing only *Actinobacillus actinomycetemcomitans* and against its planktonic variants. The three rinses produced a 99.99% reduction in planktonic forms compared to saline control. However, on the biofilm, only the rinsing of EO was effective in causing a 97% reduction in bacterial load, while the other rinses showed no significant antibacterial activity.

Posteriorly, Filoche et al. [173] performed a fluorescence assay to quantify the total viable biomass of plaque microcosms generated in an artificial mouth, under different growth conditions and after exposure to several oral antiseptics. The results showed that the viability of the 3-day samples treated with CHX or EO was not affected and that those exposed to the antiseptics from day 0 were fully recovered 14 days post-treatment.

More recently, Pan et al. [162] compared the antimicrobial activity of nine different types of rinses, including EO and CHX in an *in vitro* biofilm model where viability was assessed by bioluminescence of the bacterial ATP and CLSM. They applied the different rinses tested for 30 and 60 seconds on a biofilm of 18 hours of evolution. The application of the rinsing with EO was the only one that produced a significant reduction in the vital cellularity of the biofilm after the treatment of 30 seconds, and that caused the greater decrease in the application during 60 seconds, being 57.7% more effective than CHX.

Sliepen's group [327] evaluated the effects on viability and a total number of bacteria in established biofilms of *A. actinomycetemcomitans*, grown *in vitro*, from several rinses including EO and CHX. Two daily applications were carried out for 4 days on the different biofilms, and the CLSM was used. The authors concluded that all the tested rinses produced a reduction in the number of cultivable *A. actinomycetemcomitans*, being the CHX rinsing the most effective, both by decreasing BV and the thickness of the biofilm formed. The thickness was not affected by the EO.

Although the clinical efficacy of oral antiseptics is attributed to their high antibacterial activity, it has been shown that these may have additional effects on bacteria exposed to sublethal levels. Some authors have detected that exposure to EO can have significant effects on bacterial coagulation by decreasing bacterial multiplication and extracting endotoxins from Gram-negative bacteria by using *in vitro* models of microorganisms representative of plaque [247, 330, 331]. Lee et al. [332] observed that a particular EO (*Curcuma Longa*) exerted a significant inhibition of the adherence of *S. mutans* to saliva-coated hydroxyapatite crystals, thereby inhibiting the formation of biofilm.

- In situ activity

Charles et al. [328] determined the interproximal antibacterial efficacy of Listerine® after brushing. To that end, a total of 34 volunteers rinsed (with EO or saline negative control) after brushing. Samples of interproximal plaque were collected at 5 minutes using paper tips. The results obtained were that after a single mouthwash of EO there was a 44% reduction in BV in the interproximal spaces compared to the negative control at 5 minutes post-application.

After their *in vitro* test [326], Pan's research group [87] examined the dental plaque of 1 day of formation in 17 subjects. Using a curette, they collected the plaque from the vestibular surfaces of an upper quadrant and the lower contralateral before performing an EO mouthwash; 30 minutes after their application, they did the same with the molars and contralateral premolars. The plaque was deposited on a glass disk and stained with the fluorescence solution LIVE/DEAD® BacLight™ to differentiate living bacteria from dead, obtaining a 22.3% viability at 30 minutes, while with the negative control they got a viability of 72.1%.

Fine et al. [89] carried out a study in which they determined the antimicrobial effect of a single mouthwash of EO 12 hours after their application and after 14 days of continuous use applying plate culturing techniques. The method of plaque collection was performed with a curette. They obtained a reduction of BV that ranged from 56.3% to 87.7% concerning the negative control (5% hydroalcoholic solution) 12 hours after the rinsing application. After daily use (2 times a day/2 weeks) the percentage reduction in BV amounted to 72.5%-93.8% with respect to the negative control.

Dong et al. [174] attempted to establish an *in situ* model to collect intact dental plaque to evaluate its structure, immediate penetration and antibacterial effect of a rinsing of EO. For this, they performed cuts of 500 μm depth on hydroxyapatite disks. A total of six volunteers carried the named disks for 6, 24 and 48 hours. The disks were then divided into two halves, one immersed in an EO solution for 1 minute and the other in a saline solution. Afterwards, the dental plaque was assessed applying Live/Dead® Baclight™ in combination with CLSM at 5, 15 and 30 minutes. The thicknesses of the dental plaque at 6, 24 and 48 hours were 11.92 μm, 18.63 μm and 27.55 μm, respectively. A significant reduction in BV was also found concerning the control group.

Gosau et al. [98] evaluated the antimicrobial activity of EO on a 12-hour oral biofilm, formed on the surface of various titanium disks supported by a superior splint in a total of four volunteers. The application of the rinse was performed extraorally for 1 minute. After using the LIVE/DEAD® BacLight™ staining solution and the CLSM for visualisation of the PL-biofilm *in situ*, the percentage of dead bacteria at 30 seconds was 76.8% (range = 65.09%-95.87%).

Recently, Prada-López et al. [187] conducted a study to assess how the mode of application of the antiseptic could influence in the antibacterial activity of the antiseptic. They compared the effect of doing an active rinsing with CHX and EO with a passive immersion in these same solutions. They found that both antiseptics had considerable less effect when they were not applied as an active mouthwash. This phenomenon was especially appreciated with the EO solutions, with great differences being detected between both types of applications (BV after an active mouthwash *vs.* BV after a passive immersion = 1.16% *vs.* 26.93%, $p<0.001$). The BV found

after the mouthwashes was lower for the EO than for the CHX (1.16% *vs.* 5.08%).

I.7.2.2. IMMEDIATE ANTIBACTERIAL EFFECT AND SUBSTANTIVITY OF THE CHLORHEXIDINE ON ORAL BIOFILM

- In vitro activity

Although there are some studies assessing the effect of the CHX on monospecies biofilm [54, 55, 333-341], in the present Thesis we are only referring to the consequences of the CHX on *in vitro* multispecies biofilms.

Kinniment et al. [52] compared the antibacterial effect of CHX at different concentrations (0.0125% and 0.125%), concluding that the lower concentration was ineffective while, the higher obtained a differential and significative inhibition.

In 1998, Pratten et al. [65] exposed samples composed of six bacterial species (*Streptococcus sanguis, S. mutans, Streptococcus oralis, Actinomyces naeslundii, Neisseria subflava* and *Veillonella dispar* formed on bovine enamel disks to the effects of CHX. For this, they exposed the samples to the 0.2% CHX action for 1, 5 and 60 minutes; later, the surviving bacteria were quantified by cultures and with LIVE/DEAD® BacLight™ staining. Exposure to CHX for 1 and 5 minutes did not have a noticeable effect on the viability of any of the six species, whereas the exposure during 60 minutes produced a significant bacterial mortality, which, according to the authors, demonstrated the importance of the substantivity of antimicrobial agents. This same group [342], applying a similar methodology, assessed the impact of implementing sucrose supplementation in the development of microcosms of dental plaque. The

most remarkable result was that, despite the fact that viable bacterial counts were reduced, various microorganisms survived the CHX exposure for 1 minute while only 2-4% of the bacterial population was viable after 60 minutes of exposure to CHX.

One year later, Pratten and Wilson [58] repeated the same methodological design, to evaluate the effect of two daily sprays with 0.2% CHX digluconate on the viability and composition of plaque microcosms. The results showed that after 5 days from the last spray there was still a certain reduction in total bacterial counts, but that after 9 days the bacterial population exceeded the basal levels.

The CHX has also been used as a reference standard to evaluate *in vitro* the antibacterial efficacy of other chemical agents on multispecies biofilms. In 2001, Guggenheim et al. [343] quantified the effectiveness of CHX digluconate and triclosan. The antibacterial effect of triclosan was lower than CHX, and the response to this antiseptic was concentration-dependent (0.2% CHX reduced the viability of the biofilm to 0%). A year later, Shapiro et al. [344] also demonstrated the utility of an *in vitro* biofilm model to evaluate preclinically oral antiseptic solutions, although in this case, they tested 12 different types of mouthwash. The biofilm consisted of five bacterial species (in this case, also *Candida albicans*) and formed on hydroxyapatite disks. Their results showed that the incorporation of the fungal species did not condition the CHX efficacy and that the compounds of botanical origin, as well as the phenolics, were less effective than the majority of those containing CHX, hexetidine or octenidine.

- In situ activity

Despite a significant amount of information available on the antimicrobial activity of CHX in biofilm, only three papers have been published so far in which the effect of a single application of this antiseptic on PL-biofilm [107, 170, 187]. However, only in one of the studies the samples were not treated and processed *ex vivo*.

In 2001, Zaura-Arite et al. [170] were the first authors to quantify the antimicrobial effect of a single *ex vivo* application of CHX on PL-biofilm samples formed *in vivo* on bovine dentin disks. The disks were removed at 6 hours, 1 and 2 days, and divided into two halves through a central slot. One-half was coated for 1 minute with 50 µL of 0.2% CHX, while the other was immersed in saline and used as a control. Although there were significant inter-individual differences, the authors noted that in PL-biofilm with more than 65 µm in thickness the bactericidal effect of the CHX was small and superficial. The mean values of BV in control and CHX treated disks were: at 6 hours, 35% *vs.* 18%; in the 1-day biofilm samples, 49% *vs.* 34% in the outer layer, 67% *vs.* 42% in the middle layer, and 50% *vs.* 48% in the inner layer; in the 2-day biofilm samples, 52% *vs.* 33% in the outer layer, 63% *vs.* 42% in the middle layer, and 54% *vs.* 48% in the inner layer. During the experiment period, the participants maintained their usual diet and carried a removable disk holding intraoral appliance, which they only removed twice a day to brush their teeth. It should be noted that brushing was performed with toothpaste with sodium fluoride, which probably could condition the results [170].

In 2010, von Ohle et al. [107] analysed the effect of CHX on the physiology of PL-biofilm supplemented with sucrose. They exposed the

biofilm samples *ex vivo* for 1 and 10 minutes to 10 mL of a 0.2% solution of this antiseptic. In contrast to the results obtained by Zaura-Arite [170], CHX significantly reduced BV (67% in PL-biofilm control *vs.* 2% and 0.7% in samples exposed to CHX for 1 and 10 minutes, respectively). However, CHX did not significantly affect oxidative metabolism, since after 10 minutes of exposure the biofilm was still physiologically active, evidencing an incomplete bacterial annihilation and a functional readaptation of the deeper layers of the biofilm. A particularly relevant aspect of the contributions of these authors was that, although the BV after 1 minute of CHX exposure estimated with LIVE/DEAD® BacLight™ staining was 2%, in a conventional culture it was 58%. This finding allows assuming that after short exposures some bacteria persist cultivable even when their membranes are damaged. However, after 10 minutes of CHX exposure, the number of colony forming units/mL was significantly reduced compared to that obtained in the PL-biofilm control.

In 2015, Prada-López et al. [187] performed a study to compare the immediate antibacterial effect of two application methods of the CHX. It is the only study available that analyses the effect of a single application of CHX applied *in situ* on a PL-biofilm. The PL-biofilm was grown *in situ* on glass disks during 48 hours. After this period, the disks were either immersed in 0.2% CHX for 30 seconds (passive immersion) or kept in the mouth for doing an active mouthwash. The *in situ* application obtained a mean viability of 5%, which was significantly lower than that achieved with the passive immersion (15%). These significant differences were patent in all PL-biofilm layers. With this study, the authors showed the importance that the hydrodynamic forces of an active mouthwash may have in the capacity of the CHX to affect the BV. The thickness was also measured, but it was not affected by any of the application methods.

I.7.2.3. ANTIPLAQUE EFFECT OF THE ESSENTIAL OILS ON ORAL BIOFILM

The analysis of the impact of various applications of mouthwashes on oral biofilm is more commonly found in literature than single applications. This fact is because that mouthwashes are frequently designed and tested to be used as a complement of daily oral hygiene more than a single treatment. However, there is only one study in the literature [345] in which the authors analysed the antiplaque effect of the EO on *in situ* PL-biofilm. For this reason, apart from this one, in the present Thesis the described papers are those which analyse the effect of the EO on destructured dental plaque [81, 346] in the short time (until 4 days). Also, those papers which analyse the growth of the dental plaque on the surface of the teeth by plaque indexes [79, 319, 320, 347-349].

In 2013, Jentsch et al. [345] evaluated the effect that daily mouthwashes could have in the dental plaque formed *in situ* on the surface of enamel disks hold on acrylic splints. The volunteers rinsed twice daily with EO or 0.12% CHX. The disks were removed at 24, 48, 72 and 96 hours after the start of the regrowth period. The PL-biofilm was analysed using the SEM counting the number of cocci and bacilli as well as the dental plaque thickness. The 0.12% CHX performed better than the EO regarding reducing the number of cocci at 4 days (14.58 *vs.* 29.23 in an area of 6.25 μm^2) as well as a number of bacilli (0.13 *vs.* 2.14 in an area of 6.25 μm^2). However, there were no significant differences regarding dental plaque thickness (thickness = 16.67 µm *vs.* 15.13 µm). These results on thickness should be taken with caution because, as previously mentioned, the preparation to which the samples must be subjected can cause shrinkage, loss of extracellular matrix and lead to the appearance of artefacts [96].

In 2011, Marchetti et al. [347] used a 3-day plaque regrowth study to assess the antiplaque effect of the EO with and without alcohol. The volunteers refrained from any oral hygiene measure other than performing two daily mouthwashes with the EO either with or without alcohol. The same subjects repeated the process with the other formulation after a washout period of 2 weeks. Plaque indexes were measured at the end of both periods. The alcohol-free EO showed lower antiplaque effect than the traditional EO formulation (plaque index = 2.28 *vs.* 2.57). Recently, following the same design from the previous study, this group performed a new one [348] in which they evaluated the effect of the EO without alcohol and the 0.2% CHX. They obtained that the alcohol-free EO were less effective than the CHX (plaque index = 2.45 *vs.* 1.41) and did not get significant differences about the control. This same group performed a recent clinical trial [349] in which they compared the effects of the EO with and without alcohol. The only difference *versus* the previous study is that this time they used two EO formulas from the same manufacturer (Johnson & Johnson). In this study, they found that both EO formulas were effective at reducing the plaque levels *vs.* the negative control with a plaque index of around 1.7 for both solutions; these results were statistically lower than the control (2.3).

As previously stated, the 4-day plaque regrowth period is recognised to be predictable of the antiplaque effect of a determinate oral solution. Thus, in the literature, some studies are found assessing the effect of the EO [79, 319, 320, 324, 325, 346, 350].

In 1997, Moran et al. [319] analysed the effect of two daily EO mouthwashes for 4 days as the single oral hygiene measure used by 32 volunteers. The EO reduced the plaque accumulation by a 52%, being the

plaque index a 17% lower than the placebo. Following a similar methodology, Riep et al. [320] obtained that the EO reduced the dental plaque levels by a 23% about the placebo; the 0.1% CHX provoked a 38.2% of the reduction in this case. In 2001, Rosin et al. [79] obtained even better effects of EO getting those close to the 0.12% CHX, obtaining a plaque index of 1.8, rather lower than the placebo, which was situated in 2.6.

Some years later, the group of Pizzo [324] studied the antiplaque effects of the continuous rinsing with 0.12% CHX, amine fluoride/stannous fluoride and EO. All solutions were effective when compared to the negative control, being the 0.12% CHX the most effective (plaque index = 1.21 *vs.* 3.08) followed by the EO (plaque index = 1.91 *vs.* 3.08). Afterwards, this same group [350], performed a similar study. This time they compared the antiplaque effects of an alcohol-free EO to the traditional formulation of EO with alcohol. They found that the alcohol-free formulation was less effective than the traditional one, obtaining a similar result to the negative control (plaque index = 3.17 *vs.* 3.28). This same year, Singh et al. [325] found in a 4-day plaque regrowth study similar results to those of Pizzo et al., where EO mouthwashes were effective at reducing the plaque indexes with regard to the control (3.21 *vs*. 3.77) but CHX was more efficient (2.69 *vs.* 3.21)

Another study by Ulkur et al. [346] compared the effects of three different types of mouthwash (0.1% CHX, EO and alcohol-free EO) daily applied on the *S. mutans* population for 4 days. Using plate culturing techniques for the assessments, no significant differences were found between the three mouthwashes, being both EO formulations as effective as the 0.1% CHX. In the same line, Rosin et al. [81] assessed the efficacy of 0.12% CHX and an EO formulation by plate culture techniques. They,

again, did not find significant differences in the number of bacteria present on the surface of the teeth after 4 days when applying 0.12% CHX or EO.

I.7.2.4. ANTIPLAQUE EFFECT OF THE CHLORHEXIDINE ON ORAL BIOFILM

The antiplaque properties of the CHX have been extensively tested in models of the short and long term. This fact is because, typically, it is used as the positive control as seen in the previous section [79, 81, 320, 324, 325, 346, 350]. In addition to these previously named studies, there are plenty of *in vivo* studies measuring dental plaque indexes that test the CHX at different concentrations [67, 147, 216, 246, 252, 258, 351, 352]. In this Thesis, however, the description of them all is out of the scope since the CHX is used as a positive control. Nevertheless, it is worth to describe those studies which test the CHX in the short term on *in situ* PL-biofilm. To the best of author's knowledge, there are only four studies in the literature which test various applications of CHX on *in situ* PL-biofilm [70, 121, 345, 353]. One of them has been [345] previously described.

In 2002, Jentsch et al. [353] were the first at testing various applications of CHX *in situ*. Twenty-one volunteers wore an acrylic splint for 72 hours while they rinsed twice daily with 0.12 CHX, stannous/amine fluoride or a saline solution. After the 3-day period, the samples were analysed by TEM assessing the thickness of the PL-biofilm, as well as the number of cocci and bacilli. The obtained thickness for the control was 19.24 µm, being the thickness obtained for the CHX almost half of this number with 11.91 µm. The 0.12% CHX was the most effective at reducing the number of cocci per 10 μm^2 (4.29) concerning the results of the amine/stannous fluoride and the saline solutions (12.04 and 18.18, respectively). In regard with the bacilli, again the CHX was the most effective (0.08 *vs.* 0.52 *vs.* 1.29).

In 2005, Auschil et al. [70] assessed in seven volunteers the effects of rinsing twice daily with 0.2% CHX, amine/stannous fluoride or water for 48 hours. This time, the authors used the CLSM for analysing the thickness and BV of the *in situ* PL-biofilm. Regarding the thickness, the 0.2% CHX was the most effective with a PL-biofilm more nine times thinner than the control (8.6 µm *vs.* 76.7 µm). The BV was reduced from 66.1% for the control to a 23.3% for the 0.2% CHX. Once again, the CHX, this time at a higher concentration, showed its powerful antiplaque effect.

Later, in 2008, Arweiler et al. [121] used 24 volunteers for studying the effect of 0.1% sorbate, 0.1% benzoate, 0.2% CHX and saline solutions. The subjects wore individualised acrylic splints which held three glass disks by hemi-arch. They had to immerse twice daily for 5 days half of the splint in one of the test solutions and the other half in the saline. After the 5 days, the PL-biofilm samples were analysed by CLSM for thickness and BV. The 0.2% CHX reduced the biofilm thickness by a 57% (10.8 µm *vs.* 25.3 µm). Regarding the BV, the 0.2% CHX was again the most effective obtaining a 62% reduction (21.7% *vs.* 56.8%). In this study, there is a remarkable difference regarding the previous study from Auschil et al. [70]; it is the fact that the volunteers did not perform the mouthwashes themselves but they immersed the specimens in the test and control solutions. The available literature has revealed that an active mouthwash and a passive immersion do not have comparable antibacterial effects on the *in situ* PL-biofilm [187].

I.8. REFERENCES

1. **Overman, P.R.**, *Biofilm: a new view of plaque.* J Contemp Dent Pract, 2000. 1(3): p. 18-29.

2. **Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M.**, *Microbial biofilms.* Annu Rev Microbiol, 1995. 49: p. 711-45.

3. **Slavkin, H.C.**, *Biofilms, microbial ecology and Antoni van Leeuwenhoek.* J Am Dent Assoc, 1997. 128(4): p. 492-5.

4. **Costerton, J.W., Stewart, P.S., and Greenberg, E.P.**, *Bacterial biofilms: a common cause of persistent infections.* Science, 1999. 284(5418): p. 1318-22.

5. **Watnick, P.I. and Kolter, R.**, *Steps in the development of a Vibrio cholerae El Tor biofilm.* Mol Microbiol, 1999. 34(3): p. 586-95.

6. **Fine, D.H., Furgang, D., and Barnett, M.L.**, *Comparative antimicrobial activities of antiseptic mouthrinses against isogenic planktonic and biofilm forms of Actinobacillus actinomycetemcomitans.* J Clin Periodontol, 2001. 28(7): p. 697-700.

7. **Mack, W.N., Mack, J.P., and Ackerson, A.O.**, *Microbial film development in a trickling filter.* Microb Ecol, 1975. 2(3): p. 215-26.

8. **Donlan, R.M. and Costerton, J.W.**, *Biofilms: survival mechanisms of clinically relevant microorganisms.* Clin Microbiol Rev, 2002. 15(2): p. 167-93.

9. **Aas, J.A., Paster, B.J., Stokes, L.N., Olsen, I., and Dewhirst, F.E.**, *Defining the normal bacterial flora of the oral cavity.* J Clin Microbiol, 2005. 43(11): p. 5721-32.

10. **Manson, J.M., Rauch, M., and Gilmore, M.S.**, *The commensal microbiology of the gastrointestinal tract.* Adv Exp Med Biol, 2008. 635: p. 15-28.

11. **Peters, B.M., Jabra-Rizk, M.A., O'May, G.A., Costerton, J.W., and Shirtliff, M.E.**, *Polymicrobial interactions: impact on pathogenesis and human disease.* Clin Microbiol Rev, 2012. 25(1): p. 193-213.

12. **Marsh, P.D.**, *Microbial ecology of dental plaque and its significance in health and disease.* Adv Dent Res, 1994. 8(2): p. 263-71.

13. **Li, J., Helmerhorst, E.J., Leone, C.W., Troxler, R.F., Yaskell, T., Haffajee, A.D., Socransky, S.S., and Oppenheim, F.G.**, *Identification of early microbial colonizers in human dental biofilm.* J Appl Microbiol, 2004. 97(6): p. 1311-8.

14. **WD., M.**, *Dental plaque: thirty years on*, in *Dental plaque revisited: oral biofilms in health and disease*, W.M. Newman HN, Editor. 1999: Bioline, Cardiff. p. 1-4.

15. **Socransky, S.S. and Haffajee, A.D.**, *Dental biofilms: difficult therapeutic targets.* Periodontol 2000, 2002. 28: p. 12-55.

16. **Marsh, P.D.**, *Dental plaque as a microbial biofilm.* Caries Res, 2004. 38(3): p. 204-11.

17. **Haffajee, A.D. and Socransky, S.S.**, *Introduction to microbial aspects of periodontal biofilm communities, development and treatment.* Periodontol 2000, 2006. 42: p. 7-12.

18. **ten Cate, J.M.**, *Biofilms, a new approach to the microbiology of dental plaque.* Odontology, 2006. 94(1): p. 1-9.

19. **Black, G.V.**, *Dr. Black´s conclusions reviewed again.* Dent Cosmos, 1898. 40: p. 440-451.

20. **Gibbons, R.J. and Banghart, S.B.**, *Synthesis of extracellular dextran by cariogenic bacteria and its presence in human dental plaque.* Arch Oral Biol, 1967. 12(1): p. 11-23.

21. **Marshall, K.C.**, *Interfaces in microbial ecology*. 1976, Massachusetts: Harvard University Press.

22. **Marsh, P.D.**, *Microbiologic aspects of dental plaque and dental caries.* Dent Clin North Am, 1999. 43(4): p. 599-614, v-vi.

23. **Levine, M.J., Reddy, M.S., Tabak, L.A., Loomis, R.E., Bergey, E.J., Jones, P.C., Cohen, R.E., Stinson, M.W., and Al-Hashimi, I.**, *Structural aspects of salivary glycoproteins.* J Dent Res, 1987. 66(2): p. 436-41.

24. **Tabak, L.A., Levine, M.J., Mandel, I.D., and Ellison, S.A.**, *Role of salivary mucins in the protection of the oral cavity.* J Oral Pathol, 1982. 11(1): p. 1-17.

25. **Gibbons, R.J. and Hay, D.I.**, *Human salivary acidic proline-rich proteins and statherin promote the attachment of Actinomyces viscosus LY7 to apatitic surfaces.* Infect Immun, 1988. 56(2): p. 439-45.

26. **Hannig, C., Hannig, M., Rehmer, O., Braun, G., Hellwig, E., and Al-Ahmad, A.**, *Fluorescence microscopic visualization and quantification of initial bacterial colonization on enamel in situ.* Arch Oral Biol, 2007. 52(11): p. 1048-56.

27. **Jung, D.J., Al-Ahmad, A., Follo, M., Spitzmuller, B., Hoth-Hannig, W., Hannig, M., and Hannig, C.**, *Visualization of initial bacterial colonization on dentine and enamel in situ.* J Microbiol Methods, 2010. 81(2): p. 166-74.

28. **Al-Ahmad, A., Wiedmann-Al-Ahmad, M., Fackler, A., Follo, M., Hellwig, E., Bachle, M., Hannig, C., Han, J.S., Wolkewitz, M., and Kohal, R.**, *In vivo study of the initial bacterial adhesion on different implant materials.* Arch Oral Biol, 2013.

29. **Gurenlian, J.R.**, *The Role of Dental Plaque Biofilm in Oral Health.* JDH / Am Dent Hyg' Assoc, 2007. 81(5): p. 1-11.

30. **Rickard, A.H., Gilbert, P., High, N.J., Kolenbrander, P.E., and Handley, P.S.**, *Bacterial coaggregation: an integral process in the development of multi-species biofilms.* Trends Microbiol, 2003. 11(2): p. 94-100.

31. **Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., and Marrie, T.J.**, *Bacterial biofilms in nature and disease.* Annu Rev Microbiol, 1987. 41: p. 435-64.

32. **Gibbons, R.J.**, *Adherent interactions which may affect microbial ecology in the mouth.* J Dent Res, 1984. 63(3): p. 378-85.

33. **Whittaker, C.J., Klier, C.M., and Kolenbrander, P.E.**, *Mechanisms of adhesion by oral bacteria.* Annu Rev Microbiol, 1996. 50: p. 513-52.

34. **Wirthlin, M.R. and Armitage, G.C.**, *Dental plaque and calculus: microbial biofilms and periodontal diseases*, in *Periodontics: Medicine, Surgery and Implants*, L.F. Rose, et al., Editors. 2004, Elsevier Mosby: St. Louis.

35. **Newman, H.N. and Wilson, M.A.**, *Dental plaque revisited : oral biofilms in health and disease : proceedings of a conference held at the Royal College of Physicians*. 1999, Cardiff: Bioline.

36. **Loe, H.**, *Discussion of: methods and criteria in evaluation of gingival and periodontal response.* Int Dent J, 1970. 20(3): p. 502-4.

37. **Bowen, W.H.**, *Effects of foods on oral bacterial populations in man and animals.* J Dent Res, 1970. 49(6): p. 1276-82.

38. **Löe, H. and Schiött, C.R.**, *The effect of suppression of the oral microflora upon The formation of dental plaque*, in *Dental Plaque*, W.D. McHugh, Editor. 1970, E. S. Livingstone: Edimburgh. p. 247-255.

39. **Loe, H., Theilade, E., and Jensen, S.B.**, *Experimental Gingivitis in Man.* J Periodontol, 1965. 36: p. 177-87.

40. **Loe, H., Theilade, E., Jensen, S.B., and Schiott, C.R.**, *Experimental gingivitis in man. 3. Influence of antibiotics on gingival plaque development.* J Periodontal Res, 1967. 2(4): p. 282-9.

41. **Theilade, J., Lobene, R.R., Mandel, I.D., and Hazen, S.**, *What is the relationship between bacterial plaque and calculus formation?* Periodontal Abstr, 1967. 15(2): p. 53-6.

42. **Jensen, S.B., Loe, H., Schiott, C.R., and Theliade, E.**, *Experimental gingivitis in man. 4. Vancomycin induced changes in bacterial plaque composition as related to development of gingival inflammation.* J Periodontal Res, 1968. 3(4): p. 284-93.

43. **Loe, H. and Schiott, C.R.**, *The effect of mouthrinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man.* J Periodontal Res, 1970. 5(2): p. 79-83.

44. **Bowden, G.H. and Li, Y.H.**, *Nutritional influences on biofilm development.* Adv Dent Res, 1997. 11(1): p. 81-99.

45. **Biofilm Club, M. and Gilbert, P.**, *Biofilms : coming of age : contributions made at the eighth meeting of the Biofilm Club held at Gregynog Hall, Powys 5th-7th September 2007*. 2007, Manchester: Biofilm Club.

46. **Tatakis, D.N. and Kumar, P.S.**, *Etiology and pathogenesis of periodontal disease.* Dent Clin Nam, 2005. 49: p. 491-516.

47. **Jefferson, K.K. and Cerca, N.**, *Bacterial-bacterial cell interactions in biofilms: detection of polysaccharide intercellular adhesins by blotting and confocal microscopy.* Methods Mol Biol, 2006. 341: p. 119-26.

48. **Kolenbrander, P.E., Palmer, R.J., Jr., Rickard, A.H., Jakubovics, N.S., Chalmers, N.I., and Diaz, P.I.**, *Bacterial interactions and successions during plaque development.* Periodontol 2000, 2006. 42: p. 47-79.

49. **McBain, A.J.**, *Chapter 4: In vitro biofilm models: an overview.* Adv Appl Microbiol, 2009. 69: p. 99-132.

50. **Nobbs, A.H., Lamont, R.J., and Jenkinson, H.F.**, *Streptococcus adherence and colonization.* Microbiol Mol Biol Rev, 2009. 73(3): p. 407-50, Table of Contents.

51. **Palmer, R.J., Jr.**, *Supragingival and subgingival plaque: paradigm of biofilms.* Compend Contin Educ Dent, 2010. 31(2): p. 104-6, 108, 110 passim; quiz 124, 138.

52. **Kinniment, S.L., Wimpenny, J.W., Adams, D., and Marsh, P.D.**, *Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter.* Microbiology, 1996. 142 (Pt 3): p. 631-8.

53. **Frías, J.M.**, *Nuevas perspectivas en biofilms dentales. Temas de revisión.* Periodoncia: Sociedad Española de Periodoncia, 2001. 11: p. 23-32.

54. **Millward, T.A. and Wilson, M.**, *The effect of chlorhexidine on Streptococcus sanguis biofilms.* Microbios, 1989. 58(236-237): p. 155-64.

55. **Wilson, M.**, *Susceptibility of oral bacterial biofilms to antimicrobial agents.* J Med Microbiol, 1996. 44(2): p. 79-87.

56. **Bradshaw, D.J., McKee, A.S., and Marsh, P.D.**, *Prevention of population shifts in oral microbial communities in vitro by low fluoride concentrations.* J Dent Res, 1990. 69(2): p. 436-41.

57. **Marsh, P.D. and Bradshaw, D.J.**, *Microbiological effects of new agents in dentifrices for plaque control.* Int Dent J, 1993. 43(4 Suppl 1): p. 399-406.

58. **Pratten, J. and Wilson, M.**, *Antimicrobial susceptibility and composition of microcosm dental plaques supplemented with sucrose.* Antimicrob Agents Chemother, 1999. 43(7): p. 1595-9.

59. **Wilson, M.**, *Use of constant depth film fermentor in studies of biofilms of oral bacteria.* Methods Enzymol, 1999. 310: p. 264-79.

60. **Pratten, J., Andrews, C.S., Craig, D.Q., and Wilson, M.**, *Structural studies of microcosm dental plaques grown under different nutritional conditions.* FEMS Microbiol Lett, 2000. 189(2): p. 215-8.

61. **Hope, C.K., Clements, D., and Wilson, M.**, *Determining the spatial distribution of viable and nonviable bacteria in hydrated microcosm dental plaques by viability profiling.* J Appl Microbiol, 2002. 93(3): p. 448-55.

62. **Hope, C.K. and Wilson, M.**, *Biofilm structure and cell vitality in a laboratory model of subgingival plaque.* J Microbiol Methods, 2006. 66(3): p. 390-8.

63. **Hope, C.K. and Wilson, M.**, *Measuring the thickness of an outer layer of viable bacteria in an oral biofilm by viability mapping.* J Microbiol Methods, 2003. 54(3): p. 403-10.

64. **Pratten, J.**, *Growing oral biofilms in a constant depth film fermentor (CDFF).* Curr Protoc Microbiol, 2007. Chapter 1: p. Unit 1B 5.

65. **Pratten, J., Barnett, P., and Wilson, M.**, *Composition and susceptibility to chlorhexidine of multispecies biofilms of oral bacteria.* Appl Environ Microbiol, 1998. 64(9): p. 3515-9.

66. **Tang, G., Yip, H.K., Cutress, T.W., and Samaranayake, L.P.**, *Artificial mouth model systems and their contribution to caries research: a review.* J Dent, 2003. 31(3): p. 161-71.

67. **Sissons, C.H. and Yakub, S.**, *Suppression of urease levels in Streptococcus salivarius by cysteine, related compounds and by sulfide.* Oral Microbiol Immunol, 2000. 15(5): p. 317-24.

68. **Wecke, J., Kersten, T., Madela, K., Moter, A., Gobel, U.B., Friedmann, A., and Bernimoulin, J.**, *A novel technique for monitoring the development of bacterial biofilms in human periodontal pockets.* FEMS Microbiol Lett, 2000. 191(1): p. 95-101.

69. **Auschill, T.M., Hellwig, E., Sculean, A., Hein, N., and Arweiler, N.B.**, *Impact of the intraoral location on the rate of biofilm growth.* Clin Oral Investig, 2004. 8(2): p. 97-101.

70. **Auschill, T.M., Hein, N., Hellwig, E., Follo, M., Sculean, A., and Arweiler, N.B.**, *Effect of two antimicrobial agents on early in situ biofilm formation.* J Clin Periodontol, 2005. 32(2): p. 147-52.

71. **Watson, P.S., Pontefract, H.A., Devine, D.A., Shore, R.C., Nattress, B.R., Kirkham, J., and Robinson, C.**, *Penetration of fluoride into natural plaque biofilms.* J Dent Res, 2005. 84(5): p. 451-5.

72. **Al-Ahmad, A., Wunder, A., Auschill, T.M., Follo, M., Braun, G., Hellwig, E., and Arweiler, N.B.**, *The in vivo dynamics of Streptococcus spp., Actinomyces naeslundii, Fusobacterium nucleatum and Veillonella spp. in dental plaque biofilm as analysed by five-colour multiplex fluorescence in situ hybridization.* J Med Microbiol, 2007. 56(Pt 5): p. 681-7.

73. **Hannig, C. and Hannig, M.**, *The oral cavity--a key system to understand substratum-dependent bioadhesion on solid surfaces in man.* Clin Oral Investig, 2009. 13(2): p. 123-39.

74. **Palmer, R.J., Jr., Wu, R., Gordon, S., Bloomquist, C.G., Liljemark, W.F., Kilian, M., and Kolenbrander, P.E.**, *Retrieval of biofilms from the oral cavity.* Methods Enzymol, 2001. 337: p. 393-403.

75. **Thurnheer, T., Gmur, R., Shapiro, S., and Guggenheim, B.**, *Mass transport of macromolecules within an in vitro model of supragingival plaque.* Appl Environ Microbiol, 2003. 69(3): p. 1702-9.

76. **Stewart, P.S., Rayner, J., Roe, F., and Rees, W.M.**, *Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates.* J Appl Microbiol, 2001. 91(3): p. 525-32.

77. **Schiott, C.R., Loe, H., Jensen, S.B., Kilian, M., Davies, R.M., and Glavind, K.**, *The effect of chlorhexidine mouthrinses on the human oral flora.* J Periodontal Res, 1970. 5(2): p. 84-9.

78. **Buckner, R.Y., Kayrouz, G.A., and Briner, W.**, *Reduction of oral microbes by a single chlorhexidine rinse.* Compendium (Newtown, Pa), 1994. 15(4): p. 512, 514, 516 passim; quiz 520.

79. **Rosin, M., Welk, A., Bernhardt, O., Ruhnau, M., Pitten, F.A., Kocher, T., and Kramer, A.**, *Effect of a polyhexamethylene biguanide mouthrinse on bacterial counts and plaque.* J Clin Periodontol, 2001. 28(12): p. 1121-6.

80. **Daneshmand, N., Jorgensen, M.G., Nowzari, H., Morrison, J.L., and Slots, J.**, *Initial effect of controlled release chlorhexidine on subgingival microorganisms.* J Periodontal Res, 2002. 37(5): p. 375-9.

81. **Rosin, M., Welk, A., Kocher, T., Majic-Todt, A., Kramer, A., and Pitten, F.A.**, *The effect of a polyhexamethylene biguanide mouthrinse compared to an essential oil rinse and a chlorhexidine rinse on bacterial counts and 4-day plaque regrowth.* J Clin Periodontol, 2002. 29(5): p. 392-9.

82. **Sreenivasan, P.K., Mattai, J., Nabi, N., Xu, T., and Gaffar, A.**, *A simple approach to examine early oral microbial biofilm formation and the effects of treatments.* Oral Microbiol Immunol, 2004. 19(5): p. 297-302.

83. **Konig, J., Storcks, V., Kocher, T., Bossmann, K., and Plagmann, H.C.**, *Anti-plaque effect of tempered 0.2% chlorhexidine rinse: an in vivo study.* J Clin Periodontol, 2002. 29(3): p. 207-10.

84. **Arweiler, N.B., Boehnke, N., Sculean, A., Hellwig, E., and Auschill, T.M.**, *Differences in efficacy of two commercial 0.2% chlorhexidine mouthrinse solutions: a 4-day plaque re-growth study.* J Clin Periodontol, 2006. 33(5): p. 334-9.

85. **van der Mei, H.C., White, D.J., Atema-Smit, J., van de Belt-Gritter, E., and Busscher, H.J.**, *A method to study sustained antimicrobial activity of rinse and dentifrice components on biofilm viability in vivo.* J Clin Periodontol, 2006. 33(1): p. 14-20.

86. **Al-Ahmad, A., Maier, J., Follo, M., Spitzmuller, B., Wittmer, A., Hellwig, E., Hubner, J., and Jonas, D.**, *Food-borne enterococci integrate into oral biofilm: an in vivo study.* J Endod, 2010. 36(11): p. 1812-9.

87. **Pan, P., Barnett, M.L., Coelho, J., Brogdon, C., and Finnegan, M.B.**, *Determination of the in situ bactericidal activity of an essential oil mouthrinse using a vital stain method.* J Clin Periodontol, 2000. 27(4): p. 256-61.

88. **Tomás, I., García-Caballero, L., Cousido, M.C., Limeres, J., Álvarez, M., and Diz Dios, P.**, *Evaluation of chlorhexidine substantivity on salivary flora by epifluorescence microscopy.* Oral Dis, 2009. 15(6): p. 428-33.

89. **Fine, D.H., Furgang, D., Sinatra, K., Charles, C., McGuire, A., and Kumar, L.D.**, *In vivo antimicrobial effectiveness of an essential oil-containing mouth rinse 12 h after a single use and 14 days' use.* J Clin Periodontol, 2005. 32(4): p. 335-40.

90. **Loivukene, K., Pahkla, E.R., Koppel, T., Saag, M., and Naaber, P.**, *The microbiological status of patients with periodontitis in southern Estonia after non-surgical periodontal therapy.* Stomatologija, 2005. 7(2): p. 45-7.

91. **Wood, S.R., Kirkham, J., Marsh, P.D., Shore, R.C., Nattress, B., and Robinson, C.**, *Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy.* J Dent Res, 2000. 79(1): p. 21-7.

92. **Dige, I., Nyengaard, J.R., Kilian, M., and Nyvad, B.**, *Application of stereological principles for quantification of bacteria in intact dental biofilms.* Oral Microbiol Immunol, 2009. 24(1): p. 69-75.

93. **Arweiler, N.B., Hellwig, E., Sculean, A., Hein, N., and Auschill, T.M.**, *Individual vitality pattern of in situ dental biofilms at different locations in the oral cavity.* Caries Res, 2004. 38(5): p. 442-7.

94. **Beyth, N., Yudovin-Farber, I., Perez-Davidi, M., Domb, A.J., and Weiss, E.I.**, *Polyethyleneimine nanoparticles incorporated into resin composite cause cell death and trigger biofilm stress in vivo.* Proc Natl Acad Sci U S A, 2010. 107(51): p. 22038-43.

95. **Tomás, I., García-Caballero, L., López-Álvar, E., Suárez, M., Diz, P., and Seoane, J.**, *In Situ Chlorhexidine Substantivity on Saliva and Plaque-Like Biofilm: Influence of Circadian Rhythm.* J Periodontol, 2013. 84(11): p. 1662-72.

96. **Prada-López, I., Quintas, V., Donos, N., Suárez-Quintanilla, D., and Tomás, I.**, *Characteristics of in situ oral biofilm after 2 and 4 days of evolution.* Quintessence Int, 2015. 46(4): p. 287-298.

97. **Burgers, R., Gerlach, T., Hahnel, S., Schwarz, F., Handel, G., and Gosau, M.**, *In vivo and in vitro biofilm formation on two different titanium implant surfaces.* Clin Oral Implants Res, 2010. 21(2): p. 156-64.

98. **Gosau, M., Hahnel, S., Schwarz, F., Gerlach, T., Reichert, T.E., and Burgers, R.**, *Effect of six different peri-implantitis disinfection methods on in vivo human oral biofilm.* Clin Oral Implants Res, 2010. 21(8): p. 866-72.

99. **Giordano, C., Saino, E., Rimondini, L., Pedeferri, M.P., Visai, L., Cigada, A., and Chiesa, R.**, *Electrochemically induced anatase inhibits bacterial colonization on Titanium Grade 2 and Ti6Al4V alloy for dental and orthopedic devices.* Colloids Surf B Biointerfaces, 2011. 88(2): p. 648-55.

100. **Rimondini, L., Fare, S., Brambilla, E., Felloni, A., Consonni, C., Brossa, F., and Carrassi, A.**, *The effect of surface roughness on early in vivo plaque colonization on titanium.* J Periodontol, 1997. 68(6): p. 556-62.

101. **Nyvad, B. and Fejerskov, O.**, *Scanning electron microscopy of early microbial colonization of human enamel and root surfaces in vivo.* Scand J Dent Res, 1987. 95(4): p. 287-96.

102. **Nyvad, B. and Fejerskov, O.**, *Transmission electron microscopy of early microbial colonization of human enamel and root surfaces in vivo.* Scand J Dent Res, 1987. 95(4): p. 297-307.

103. **Nyvad, B. and Kilian, M.**, *Microbiology of the early colonization of human enamel and root surfaces in vivo.* Scand J Dent Res, 1987. 95(5): p. 369-80.

104. **Chalmers, N.I., Palmer, R.J., Jr., Du-Thumm, L., Sullivan, R., Shi, W., and Kolenbrander, P.E.**, *Use of quantum dot luminescent probes to achieve single-cell resolution of human oral bacteria in biofilms.* Appl Environ Microbiol, 2007. 73(2): p. 630-6.

105. **Jenkins, S., Addy, M., and Wade, W.**, *The mechanism of action of chlorhexidine. A study of plaque growth on enamel inserts in vivo.* J Clin Periodontol, 1988. 15(7): p. 415-24.

106. **Wood, S.R., Kirkham, J., Shore, R.C., Brookes, S.J., and Robinson, C.**, *Changes in the structure and density of oral plaque biofilms with increasing plaque age.* FEMS Microbiol Ecol, 2002. 39(3): p. 239-44.

107. **von Ohle, C., Gieseke, A., Nistico, L., Decker, E.M., DeBeer, D., and Stoodley, P.**, *Real-time microsensor measurement of local metabolic activities in ex vivo dental biofilms exposed to sucrose and treated with chlorhexidine.* Appl Environ Microbiol, 2010. 76(7): p. 2326-34.

108. **Palmer, R.J., Jr., Gordon, S.M., Cisar, J.O., and Kolenbrander, P.E.**, *Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque.* J Bacteriol, 2003. 185(11): p. 3400-9.

109. **Netuschil, L., Reich, E., Unteregger, G., Sculean, A., and Brecx, M.**, *A pilot study of confocal laser scanning microscopy for the assessment of undisturbed dental plaque vitality and topography.* Arch Oral Biol, 1998. 43(4): p. 277-85.

110. **Robinson, C., Kirkham, J., Percival, R., Shore, R.C., Bonass, W.A., Brookes, S.J., Kusa, L., Nakagaki, H., Kato, K., and Nattress, B.**, *A method for the quantitative site-specific study of the biochemistry within dental plaque biofilms formed in vivo.* Caries Res, 1997. 31(3): p. 194-200.

111. **Robinson, C., Strafford, S., Rees, G., Brookes, S.J., Kirkham, J., Shore, R.C., Watson, P.S., and Wood, S.**, *Plaque biofilms: the effect of chemical environment on natural human plaque biofilm architecture.* Arch Oral Biol, 2006. 51(11): p. 1006-14.

112. **Nyvad, B. and Fejerskov, O.**, *Structure of dental plaque and the plaque-enamel interface in human experimental caries.* Caries Res, 1989. 23(3): p. 151-8.

113. **Diaz, P.I., Chalmers, N.I., Rickard, A.H., Kong, C., Milburn, C.L., Palmer, R.J., Jr., and Kolenbrander, P.E.**, *Molecular characterization of subject-specific oral microflora during initial colonization of enamel.* Appl Environ Microbiol, 2006. 72(4): p. 2837-48.

114. **Hannig, C., Basche, S., Burghardt, T., Al-Ahmad, A., and Hannig, M.**, *Influence of a mouthwash containing hydroxyapatite microclusters on bacterial adherence in situ.* Clin Oral Investig, 2013. 17(3): p. 805-14.

115. **Hannig, C., Follo, M., Hellwig, E., and Al-Ahmad, A.**, *Visualization of adherent micro-organisms using different techniques.* J Med Microbiol, 2010. 59(Pt 1): p. 1-7.

116. **Hannig, C., Kirsch, J., Al-Ahmad, A., Kensche, A., Hannig, M., and Kummerer, K.**, *Do edible oils reduce bacterial colonization of enamel in situ?* Clin Oral Investig, 2013. 17(2): p. 649-58.

117. **Hannig, M.**, *Transmission electron microscopic study of in vivo pellicle formation on dental restorative materials.* Eur J Oral Sci, 1997. 105(5 Pt 1): p. 422-33.

118. **Hannig, M.**, *Transmission electron microscopy of early plaque formation on dental materials in vivo.* Eur J Oral Sci, 1999. 107(1): p. 55-64.

119. **Hannig, M., Kriener, L., Hoth-Hannig, W., Becker-Willinger, C., and Schmidt, H.**, *Influence of nanocomposite surface coating on biofilm formation in situ.* J Nanosci Nanotechnol, 2007. 7(12): p. 4642-8.

120. **Arweiler, N.B., Henning, G., Reich, E., and Netuschil, L.**, *Effect of an amine-fluoride-triclosan mouthrinse on plaque regrowth and biofilm vitality.* J Clin Periodontol, 2002. 29(4): p. 358-63.

121. **Arweiler, N.B., Lenz, R., Sculean, A., Al-Ahmad, A., Hellwig, E., and Auschill, T.M.**, *Effect of food preservatives on in situ biofilm formation.* Clin Oral Investig, 2008. 12(3): p. 203-8.

122. **Arweiler, N.B., Netuschil, L., and Reich, E.**, *Alcohol-free mouthrinse solutions to reduce supragingival plaque regrowth and vitality. A controlled clinical study.* J Clin Periodontol, 2001. 28(2): p. 168-74.

123. **Auschill, T.M., Arweiler, N.B., Netuschil, L., Brecx, M., Reich, E., and Sculean, A.**, *Spatial distribution of vital and dead microorganisms in dental biofilms.* Arch Oral Biol, 2001. 46(5): p. 471-6.

124. **Sreenivasan, P.K., Furgang, D., Markowitz, K., McKiernan, M., Tischio-Bereski, D., Devizio, W., and Fine, D.**, *Clinical anti-microbial efficacy of a new zinc citrate dentifrice.* Clinl Oral Investig, 2009. 13(2): p. 195-202.

125. **Fine, D.H., Furgang, D., Steinberg, L.M., Olshan, A.M., Kohut, R.E., Coelho, J.F., and Harper, D.S.**, *A model for clinical evaluation of anti-microbial effects of agents on plaque colonization.* Am J Dent, 2000. 13(3): p. 153-8.

126. **Leonhardt, A., Olsson, J., and Dahlen, G.**, *Bacterial colonization on titanium, hydroxyapatite, and amalgam surfaces in vivo.* J Dent Res, 1995. 74(9): p. 1607-12.

127. **Dige, I., Nilsson, H., Kilian, M., and Nyvad, B.**, *In situ identification of streptococci and other bacteria in initial dental biofilm by confocal laser scanning microscopy and fluorescence in situ hybridization.* Eur J Oral Sci, 2007. 115(6): p. 459-67.

128. **Dige, I., Raarup, M.K., Nyengaard, J.R., Kilian, M., and Nyvad, B.**, *Actinomyces naeslundii in initial dental biofilm formation.* Microbiology, 2009. 155(Pt 7): p. 2116-26.

129. **Gu, H., Fan, D., Gao, J., Zou, W., Peng, Z., Zhao, Z., Ling, J., and LeGeros, R.Z.**, *Effect of ZnCl2 on plaque growth and biofilm vitality.* Arch Oral Biol, 2012. 57(4): p. 369-75.

130. **Grossner-Schreiber, B., Teichmann, J., Hannig, M., Dorfer, C., Wenderoth, D.F., and Ott, S.J.**, *Modified implant surfaces show different biofilm compositions under in vivo conditions.* Clin Oral Implants Res, 2009. 20(8): p. 817-26.

131. **Prada-López, I., Quintas, V., and Tomás, I.**, *The intraoral device of overlaid disk-holding splints as a new in situ oral biofilm model.* J Clin Exp Dent, 2015. 7(1): p. e126-32.

132. **Ahrens, G.**, *Effect of fluoride tablets on uptake and loss of fluoride in superficial enamel in vivo.* Caries Res, 1976. 10(2): p. 85-95.

133. **Hannig, C., Ruggeri, A., Al-Khayer, B., Schmitz, P., Spitzmuller, B., Deimling, D., Huber, K., Hoth-Hannig, W., Bowen, W.H., and Hannig, M.**, *Electron microscopic detection and activity of glucosyltransferase B, C, and D in the in situ formed pellicle.* Arch Oral Biol, 2008. 53(11): p. 1003-10.

134. **García-Caballero, L., Quintas, V., Prada-López, I., Seoane, J., Donos, N., and Tomás, I.**, *Chlorhexidine substantivity on salivary flora and plaque-like biofilm: an in situ model.* PLoS One, 2013. 8(12): p. e83522.

135. **Kato, K., Sato, T., Takahashi, N., Fukui, K., Yamamoto, K., and Nakagaki, H.**, *A method for mapping the distribution pattern of cariogenic streptococci within dental plaque in vivo.* Caries Res, 2004. 38(5): p. 448-53.

136. **He, Y., Peterson, B.W., Jongsma, M.A., Ren, Y., Sharma, P.K., Busscher, H.J., and van der Mei, H.C.**, *Stress relaxation analysis facilitates a quantitative approach towards antimicrobial penetration into biofilms.* PLoS One, 2013. 8(5): p. e63750.

137. **Pessan, J.P., Al-Ibrahim, N.S., Buzalaf, M.A., and Toumba, K.J.**, *Slow-release fluoride devices: a literature review.* J Appl Oral Sci, 2008. 16(4): p. 238-46.

138. **Prada-López, I., Quintas, V., Vilaboa, C., Suárez-Quintanilla, D., and Tomás, I.**, *Devices for in situ development of non-disturbed oral biofilm. A Systematic Review.* Front Microbiol, 2016. 7: p. 1055.

139. **Listgarten, M.A., Mayo, H.E., and Tremblay, R.**, *Development of dental plaque on epoxy resin crowns in man. A light and electron microscopic study.* J Periodontol, 1975. 46(1): p. 10-26.

140. **Listgarten, M.A.**, *Structure of the microbial flora associated with periodontal health and disease in man. A light and electron microscopic study.* J Periodontol, 1976. 47(1): p. 1-18.

141. **Handley, P.S., Sutton, N.A., and Hughes, N.**, *Problems associated with electron microscopy of biofilms*, in *Bacterial biofilms and their control in medicine and industry*, J. Wimpenny, et al., Editors. 1993, Bio-line: Cardiff. p. 61-66.

142. **Van Der Voort, H.T.M., Valkenburg, J.A.C., Woldringh, C.L., and Brakenhoff, G.J.**, *Confocal microscopy in comparison with electron and conventional light microscopy*, in *Correlative microscopy in biology: instrumentation and methods*, M. Hayat, Editor. 2012, Academic Press Inc.: London. p. 60-81.

143. **Konishi, N., Torii, Y., Kurosaki, A., Takatsuka, T., Itota, T., and Yoshiyama, M.**, *Confocal laser scanning microscopic analysis of early plaque formed on resin composite and human enamel.* J Oral Rehabil, 2003. 30(8): p. 790-5.

144. **Van Oostveldt, P. and Baumens, S.**, *Quantitative fluorescence in confocal microscopy. The effect of the detection pinhole aperture on the reabsorption and inner filter phenomena.* J Microsc, 1990. 158: p. 121-132.

145. **Visser, T.D., Groen, F.C.A., and Brakenhoff, G.J.**, *Absorption and scattering correction in fluorescence confocal microscopy.* J Microsc, 1991. 163: p. 189-200.

146. **Paramonova, E., de Jong, E.D., Krom, B.P., van der Mei, H.C., Busscher, H.J., and Sharma, P.K.**, *Low-load compression testing: a novel way of measuring biofilm thickness.* Appl Environ Microbiol, 2007. 73(21): p. 7023-8.

147. **Quintas, V., Prada-López, I., and Tomás Carmona, I.**, *Analyzing the oral biofilm using fluorescence-based microscopy: what's in a dye?*, in *Microscopy: advances in scientific research and education*, A. Méndez-Vilas, Editor. 2014, Formatex: Badajoz (Spain). p. 226-238.

148. **Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.U., and Egli, T.**, *Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry.* Appl Environ Microbiol, 2007. 73(10): p. 3283-90.

149. **Biggerstaff, J.P., Le Puil, M., Weidow, B.L., Prater, J., Glass, K., Radosevich, M., and White, D.C.**, *New methodology for viability testing in environmental samples.* Mol Cell Probes, 2006. 20(2): p. 141-6.

150. **Van den Driessche, F., Rigole, P., Brackman, G., and Coenye, T.**, *Optimization of resazurin-based viability staining for quantification of microbial biofilms.* J Microbiol Methods, 2014. 98: p. 31-4.

151. **Freitas, A.I., Vasconcelos, C., Vilanova, M., and Cerca, N.**, *Optimization of an automatic counting system for the quantification of Staphylococcus epidermidis cells in biofilms.* J Basic Microbiol, 2013.

152. **Shen, Y., Stojicic, S., and Haapasalo, M.**, *Bacterial viability in starved and revitalized biofilms: comparison of viability staining and direct culture.* J Endod, 2010. 36(11): p. 1820-3.

153. **Al-Ahmad, A., Follo, M., Selzer, A.C., Hellwig, E., Hannig, M., and Hannig, C.**, *Bacterial colonization of enamel in situ investigated using fluorescence in situ hybridization.* J Med Microbiol, 2009. 58(Pt 10): p. 1359-66.

154. **Thurnheer, T., Gmur, R., Giertsen, E., and Guggenheim, B.**, *Automated fluorescent in situ hybridization for the specific detection and quantification of oral streptococci in dental plaque.* J Microbiol Methods, 2001. 44(1): p. 39-47.

155. **Decker, E.M.**, *The ability of direct fluorescence-based, two-colour assays to detect different physiological states of oral streptococci.* Lett Appl Microbiol, 2001. 33(3): p. 188-92.

156. **Schaudinn, C., Carr, G., Gorur, A., Jaramillo, D., Costerton, J.W., and Webster, P.**, *Imaging of endodontic biofilms by combined microscopy (FISH/cLSM - SEM).* J Microsc, 2009. 235(2): p. 124-7.

157. **Al-Ahmad, A., Wiedmann-Al-Ahmad, M., Faust, J., Bachle, M., Follo, M., Wolkewitz, M., Hannig, C., Hellwig, E., Carvalho, C., and Kohal, R.**, *Biofilm formation and composition on different implant materials in vivo.* J Biomed Mater Res B Appl Biomater, 2010. 95(1): p. 101-9.

158. **Dongari-Bagtzoglou, A.**, *Pathogenesis of mucosal biofilm infections: challenges and progress.* Expert Rev Anti Infect Ther, 2008. 6(2): p. 201-8.

159. **Gu, F., Lux, R., Du-Thumm, L., Stokes, I., Kreth, J., Anderson, M.H., Wong, D.T., Wolinsky, L., Sullivan, R., and Shi, W.**, *In situ and non-invasive detection of specific bacterial species in oral biofilms using fluorescently labeled monoclonal antibodies.* J Microbiol Methods, 2005. 62(2): p. 145-60.

160. **Shapiro, H.M.**, *Flow cytometry of bacterial membrane potential and permeability.* Methods Mol Med, 2008. 142: p. 175-86.

161. **Al-Ahmad, A., Wiedmann-Al-Ahmad, M., Auschill, T.M., Follo, M., Braun, G., Hellwig, E., and Arweiler, N.B.**, *Effects of commonly used food preservatives on biofilm formation of Streptococcus mutans in vitro.* Arch Oral Biol, 2008. 53(8): p. 765-72.

162. **Pan, P.C., Harper, S., Ricci-Nittel, D., Lux, R., and Shi, W.**, *In-vitro evidence for efficacy of antimicrobial mouthrinses.* J Dent, 2010. 38 Suppl 1: p. S16-20.

163. **Coder, D.M.**, *Assessment of cell viability.* Curr Protoc Cytom, 2001. 15(9.2): p. 1-14.

164. **Cassina, V., Seruggia, D., Beretta, G.L., Salerno, D., Brogioli, D., Manzini, S., Zunino, F., and Mantegazza, F.**, *Atomic force microscopy study of DNA conformation in the presence of drugs.* Eur Biophys J, 2011. 40(1): p. 59-68.

165. **Monaco, R.R.**, *Capture of a transition state using molecular dynamics: creation of an intercalation site in dsDNA with ethidium cation.* J Nucleic Acids, 2010. 2010.

166. **Olmsted, J., 3rd and Kearns, D.R.**, *Mechanism of ethidium bromide fluorescence enhancement on binding to nucleic acids.* Biochemistry, 1977. 16(16): p. 3647-54.

167. **Netuschil, L.**, *[Vital staining of plaque microorganisms using fluorescein diacetate and ethidium bromide].* Dtsch Zahnarztl Z, 1983. 38(10): p. 914-7.

168. **Peak, E., Chalmers, I.W., and Hoffmann, K.F.**, *Development and validation of a quantitative, high-throughput, fluorescent-based bioassay to detect schistosoma viability.* PLoS Negl Trop Dis, 2010. 4(7): p. e759.

169. **Breeuwer, P., Drocourt, J.L., Bunschoten, N., Zwietering, M.H., Rombouts, F.M., and Abee, T.**, *Characterization of uptake and hydrolysis of fluorescein diacetate and carboxyfluorescein diacetate by intracellular esterases in Saccharomyces cerevisiae, which result in accumulation of fluorescent product.* Appl Environ Microbiol, 1995. 61(4): p. 1614-9.

170. **Zaura-Arite, E., van Marle, J., and ten Cate, J.M.**, *Conofocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm.* J Dent Res, 2001. 80(5): p. 1436-40.

171. **Fuchslocher Hellemann, C., Grade, S., Heuer, W., Dittmer, M.P., Stiesch, M., Schwestka-Polly, R., and Demling, A.P.**, *Three-dimensional analysis of initial biofilm formation on polytetrafluoroethylene in the oral cavity.* J Orofac Orthop, 2013.

172. **Tawakoli, P.N., Al-Ahmad, A., Hoth-Hannig, W., Hannig, M., and Hannig, C.**, *Comparison of different live/dead stainings for detection and quantification of adherent microorganisms in the initial oral biofilm.* Clin Oral Investig, 2013. 17(3): p. 841-50.

173. **Filoche, S.K., Coleman, M.J., Angker, L., and Sissons, C.H.**, *A fluorescence assay to determine the viable biomass of microcosm dental plaque biofilms.* J Microbiol Methods, 2007. 69(3): p. 489-96.

174. **Dong, W.L., Zhou, Y.H., Li, C.Z., Liu, H., Shang, S.H., and Pan, B.Q.**, *[Establishment and application of an intact natural model of human dental plaque biofilm].* Shanghai Kou Qiang Yi Xue, 2010. 19(2): p. 196-201.

175. **Boulos, L., Prevost, M., Barbeau, B., Coallier, J., and Desjardins, R.**, *LIVE/DEAD BacLight : application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water.* J Microbiol Methods, 1999. 37(1): p. 77-86.

176. **Neilands, J., Troedsson, U., Sjodin, T., and Davies, J.R.**, *The effect of delmopinol and fluoride on acid adaptation and acid production in dental plaque biofilms.* Arch Oral Biol, 2014. 59(3): p. 318-23.

177. **Cousido, M.C., Tomás Carmona, I., García-Caballero, L., Limeres, J., Álvarez, M., and Diz, P.**, *In vivo substantivity of 0.12% and 0.2% chlorhexidine mouthrinses on salivary bacteria.* Clin Oral Investig, 2010. 14(4): p. 397-402.

178. **Tomás, I., Cousido, M.C., García-Caballero, L., Rubido, S., Limeres, J., and Diz, P.**, *Substantivity of a single chlorhexidine mouthwash on salivary flora: influence of intrinsic and extrinsic factors.* J Dent, 2010. 38(7): p. 541-6.

179. **Singh, R., Stine, O.C., Smith, D.L., Spitznagel, J.K., Jr., Labib, M.E., and Williams, H.N.**, *Microbial diversity of biofilms in dental unit water systems.* Appl Environ Microbiol, 2003. 69(6): p. 3412-20.

180. **Yang, H., Acker, J., Chen, A., and McGann, L.**, *In situ assessment of cell viability.* Cell Transplant, 1998. 7(5): p. 443-51.

181. **Liminga, G., Jonsson, B., Nygren, P., and Larsson, R.**, *On the mechanism underlying calcein-induced cytotoxicity.* Eur J Pharmacol, 1999. 383(3): p. 321-9.

182. **Beletsky, I.P. and Umansky, S.R.**, *A new assay for cell death.* J Immunol Methods, 1990. 134(2): p. 201-5.

183. **López-Amoros, R., Comas, J., and Vives-Rego, J.**, *Flow cytometric assessment of Escherichia coli and Salmonella typhimurium starvation-survival in seawater using rhodamine 123, propidium iodide, and oxonol.* Appl Environ Microbiol, 1995. 61(7): p. 2521-6.

184. **Netuschil, L., Auschill, T.M., Sculean, A., and Arweiler, N.B.**, *Confusion over live/dead stainings for the detection of vital microorganisms in oral biofilms - which stain is suitable?* BMC Oral Health, 2014. 14: p. 2.

185. **Netuschil, L., Reich, E., and Brecx, M.**, *Direct measurement of the bactericidal effect of chlorhexidine on human dental plaque.* J Clin Periodontol, 1989. 16(8): p. 484-8.

186. **Weiger, R., Decker, E.M., Krastl, G., and Brecx, M.**, *Deposition and retention of vital and dead Streptococcus sanguinis cells on glass surfaces in a flow-chamber system.* Arch Oral Biol, 1999. 44(8): p. 621-8.

187. **Prada-López, I., Quintas, V., Casares-De-Cal, M.A., Suárez-Quintanilla, J.A., Suárez-Quintanilla, D., and Tomás, I.**, *Ex vivo vs. in vivo antibacterial activity of two antiseptics on oral biofilm.* Front Microbiol, 2015. 6: p. 655.

188. **Quintas, V., Prada-López, I., Donos, N., Suárez-Quintanilla, D., and Tomás, I.**, *In situ neutralisation of the antibacterial effect of 0.2% Chlorhexidine on salivary microbiota: Quantification of substantivity.* Arch Oral Biol, 2015. 60(8): p. 1109-16.

189. **Peeters, E., Nelis, H.J., and Coenye, T.**, *Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates.* J Microbiol Methods, 2008. 72(2): p. 157-65.

190. **Berg, J.D., Roberts, P.V., and Martin, A.**, *Growth of disinfection-resistant bacteria and simulation of natural aquatic environments in the chemostat.* Water Chlor Chem Envieron Impact Health Effects, 1985. 4: p. 1137-1147.

191. **Roszak, D.B. and Colwell, R.R.**, *Survival strategies of bacteria in the natural environment.* Microbiol Rev, 1987. 51(3): p. 365-79.

192. **Amy, P.S., Pauling, C., and Morita, R.Y.**, *Recovery from nutrient starvation by a marine Vibrio sp.* Appl Environ Microbiol, 1983. 45(5): p. 1685-90.

193. **Marsh, P.D.**, *Dental plaque: biological significance of a biofilm and community life-style.* J Clin Periodontol, 2005. 32 Suppl 6: p. 7-15.

194. **Costerton, J.W., Lewandowski, Z., DeBeer, D., Caldwell, D., Korber, D., and James, G.**, *Biofilms, the customized microniche.* J Bacteriol, 1994. 176(8): p. 2137-42.

195. **Thomas, J.G. and Nakaishi, L.A.**, *Managing the complexity of a dynamic biofilm.* J Am Dent Assoc, 2006. 137 Suppl: p. 10S-15S.

196. **Netuschil, L., Weiger, R., Preisler, R., and Brecx, M.**, *Plaque bacteria counts and vitality during chlorhexidine, meridol and listerine mouthrinses.* Eur J Oral Sci, 1995. 103(6): p. 355-61.

197. **Weiger, R., Netuschil, L., von Ohle, C., and Brecx, M.**, *Microbial vitality of supragingival dental plaque during initial stages of experimental gingivitis in humans.* J Periodontal Res, 1995. 30(3): p. 204-9.

198. **Weiger, R., von Ohle, C., Decker, E., Axmann-Krcmar, D., and Netuschil, L.**, *Vital microorganisms in early supragingival dental plaque and in stimulated human saliva.* J Periodontal Res, 1997. 32(2): p. 233-40.

199. **Scannapieco, F.A.**, *Saliva-bacterium interactions in oral microbial ecology.* Crit Rev Oral Biol Med, 1994. 5(3-4): p. 203-48.

200. **Kage, A. and Bernimoulin, J.P.**, *4th European symposium on the application of saliva in clinical practice and research.* Eur J Oral Sci, 1995. 103: p. S1-S41.

201. **Tenovuo, J.**, *Antimicrobial function of human saliva--how important is it for oral health?* Acta Odontol Scand, 1998. 56(5): p. 250-6.

202. **Wimpenny, J.**, *Biofilms: structure and organisation.* Microb Ecol Health Dis, 1995. 8: p. 305-308.

203. **Lawrence, J.R., Korber, D.R., Hoyle, B.D., Costerton, J.W., and Caldwell, D.E.**, *Optical sectioning of microbial biofilms.* J Bacteriol, 1991. 173(20): p. 6558-67.

204. **Singleton, S., Treloar, R., Warren, P., Watson, G.K., Hodgson, R., and Allison, C.**, *Methods for microscopic characterization of oral biofilms: analysis of colonization, microstructure, and molecular transport phenomena.* Adv Dent Res, 1997. 11(1): p. 133-49.

205. **de Beer, D. and Stoodley, P.**, *Relation between the structure of an aerobic biofilm and transport phenomena.* Water Sci Technol, 1995. 32: p. 11-18.

206. **ten Cate, J.M. and Marsh, P.D.**, *Procedures for establishing efficacy of antimicrobial agents for chemotherapeutic caries prevention.* J Dent Res, 1994. 73(3): p. 695-703.

207. **Newman, H.N.**, *Art and science, culture and dental research.* J Dent Res, 1996. 75(1): p. 526-7.

208. **Brown, M.R. and Gilbert, P.**, *Sensitivity of biofilms to antimicrobial agents.* J Appl Bacteriol, 1993. 74 Suppl: p. 87S-97S.

209. **Gilbert, P., Das, J., and Foley, I.**, *Biofilm susceptibility to antimicrobials.* Adv Dent Res, 1997. 11(1): p. 160-7.

210. **Slomka, V., Hernandez-Sanabria, E., Herrero, E.R., Zaidel, L., Bernaerts, K., Boon, N., Quirynen, M., and Teughels, W.**, *Nutritional stimulation of commensal oral bacteria suppresses pathogens: the prebiotic concept.* J Clin Periodontol, 2017.

211. **Masdea, L., Kulik, E.M., Hauser-Gerspach, I., Ramseier, A.M., Filippi, A., and Waltimo, T.**, *Antimicrobial activity of Streptococcus salivarius K12 on bacteria involved in oral malodour.* Arch Oral Biol, 2012. 57(8): p. 1041-7.

212. **Jiang, Q., Stamatova, I., Kari, K., and Meurman, J.H.**, *Inhibitory activity in vitro of probiotic lactobacilli against oral Candida under different fermentation conditions.* Benef Microbes, 2015. 6(3): p. 361-8.

213. **Nishihara, T., Suzuki, N., Yoneda, M., and Hirofuji, T.**, *Effects of Lactobacillus salivarius-containing tablets on caries risk factors: a randomized open-label clinical trial.* BMC Oral Health, 2014. 14: p. 110.

214. **Schwendicke, F., Korte, F., Dorfer, C.E., Kneist, S., Fawzy El-Sayed, K., and Paris, S.**, *Inhibition of Streptococcus mutans Growth and Biofilm Formation by Probiotics in vitro.* Caries Res, 2017. 51(2): p. 87-95.

215. **Laleman, I., Yilmaz, E., Ozcelik, O., Haytac, C., Pauwels, M., Herrero, E.R., Slomka, V., Quirynen, M., Alkaya, B., and Teughels, W.**, *The effect of a streptococci containing probiotic in periodontal therapy: a randomized controlled trial.* J Clin Periodontol, 2015. 42(11): p. 1032-41.

216. **Gibson, G.R., Hutkins, R., Sanders, M.E., Prescott, S.L., Reimer, R.A., Salminen, S.J., Scott, K., Stanton, C., Swanson, K.S., Cani, P.D., Verbeke, K., and Reid, G.**, *Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics.* Nat Rev Gastroenterol Hepatol, 2017. 14(8): p. 491-502.

217. **McDonnell, G. and Russell, A.D.**, *Antiseptics and disinfectants: activity, action, and resistance.* Clin Microbiol Rev, 1999. 12(1): p. 147-79.

218. **Tomás, I., Cousido, M.C., Tomás, M., Limeres, J., García-Caballero, L., and Diz, P.**, *In vivo bactericidal effect of 0.2% chlorhexidine but not 0.12% on salivary obligate anaerobes.* Arch Oral Biol, 2008. 53(12): p. 1186-91.

219. **Stewart, P.S.**, *Diffusion in biofilms.* J Bacteriol, 2003. 185(5): p. 1485-91.

220. **Stewart, P.S.**, *A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms.* Biotechnol Bioeng, 1998. 59(3): p. 261-72.

221. **Tatevossian, A.**, *The effects of heat inactivation, tortuosity, extracellular polyglucan and ion-exchange sites on the diffusion of [14C]-sucrose in human dental plaque residue in vitro.* Arch Oral Biol, 1985. 30(4): p. 365-71.

222. **Baker, K.A.**, *The role of dental professionals and the patient in plaque control.* Periodontol 2000, 1995. 8: p. 108-13.

223. **Chemat, F., Vian, M.A., and Cravotto, G.**, *Green extraction of natural products: concept and principles.* Int J Mol Sci, 2012. 13(7): p. 8615-27.

224. **Fischman, S.L.**, *The history of oral hygiene products: how far have we come in 6000 years?* Periodontol 2000, 1997. 15: p. 7-14.

225. **Company, L.F.** *Listerine Label of 1885*. 1912.

226. **Fine, D.H.**, *Listerine: past, present and future--a test of thyme.* J Dent, 2010. 38 Suppl 1: p. S2-5.

227. **Vlachojannis, C., Winsauer, H., and Chrubasik, S.**, *Effectiveness and Safety of a Mouthwash Containing Essential Oil Ingredients.* Phytother Res, 2012.

228. **Mandel, I.D.**, *Chemotherapeutic agents for controlling plaque and gingivitis.* J Clin Periodontol, 1988. 15(8): p. 488-98.

229. **Therapeutics, C.o.D.**, *Guidelines for acceptance of chemotherapeutic agents for the control of supragringival plaque and gingivitis.* J Am Dent Assoc, 1986(112): p. 529-532.

230. **Fornell, J., Sundin, Y., and Lindhe, J.**, *Effect of listerine on dental plaque and gingivitis.* Scand J Dent Res, 1975. 83(1): p. 18-25.

231. **Lamster IB, A.M., Seiger MC, Gordon JM.**, *The effect of Listerine Antiseptic on reduction of existing plaque and gingivitis.* Clin Prev Dent, 1983. 5: p. 12-16.

232. **Fine, D.H., Letizia, J., and Mandel, I.D.**, *The effect of rinsing with Listerine antiseptic on the properties of developing dental plaque.* J Clin Periodontol, 1985. 12(8): p. 660-6.

233. **Gordon, J.M., Lamster, I.B., and Seiger, M.C.**, *Efficacy of Listerine antiseptic in inhibiting the development of plaque and gingivitis.* J Clin Periodontol, 1985. 12(8): p. 697-704.

234. **Axelsson, P.**, *Preventive Materials, Methods, and Programs*. 2004, Quintessence: Surrey, U.K. p. 184.

235. **Administration, F.a.D.**, *Oral health care drug products for over-the-counter human use; Antigingivitis/ Antiplaque Drug Products; Establishment of a Monograph; Propoused Rules Parte III*, in *21 CFR*, F.a.D. Administration, Editor. 2003: Department of Health and Human Services.

236. **Prakash, O., Sahoo, D., and Rout, P.K.**, *Liquid CO2 extraction of Jasminum grandiflorum and comparison with conventional processes.* Nat Prod Commun, 2012. 7(1): p. 89-92.

237. **Burt, S.A., van der Zee, R., Koets, A.P., de Graaff, A.M., van Knapen, F., Gaastra, W., Haagsman, H.P., and Veldhuizen, E.J.**, *Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in Escherichia coli O157:H7.* Appl Environ Microbiol, 2007. 73(14): p. 4484-90.

238. **Hammer, K.A., Carson, C.F., and Riley, T.V.**, *Antimicrobial activity of essential oils and other plant extracts.* J Appl Microbiol, 1999. 86(6): p. 985-90.

239. **Scorzoni L, B.T., Almeida AMF, Silva DHS, Bolzani VS, Gianinni MJSM**, *The use of standar methodology for determination of antifungical activity of natural products against medical yeasts Candida spp. and Crytococcus spp.* Braz J Microbiol, 2007. 38: p. 391-397.

240. **Kalemba, D. and Kunicka, A.**, *Antibacterial and antifungal properties of essential oils.* Curr Med Chem, 2003. 10(10): p. 813-29.

241. **Burt, S.**, *Essential oils: their antibacterial properties and potential applications in foods--a review.* Int J Food Microbiol, 2004. 94(3): p. 223-53.

242. **Faleiro, L., Miguel, G., Gomes, S., Costa, L., Venancio, F., Teixeira, A., Figueiredo, A.C., Barroso, J.G., and Pedro, L.G.**, *Antibacterial and antioxidant activities of essential oils isolated from Thymbra capitata L. (Cav.) andOriganum vulgare L.* J Agric Food Chem, 2005. 53(21): p. 8162-8.

243. **Stoeken, J.E., Paraskevas, S., and van der Weijden, G.A.**, *The long-term effect of a mouthrinse containing essential oils on dental plaque and gingivitis: a systematic review.* J Periodontol, 2007. 78(7): p. 1218-28.

244. **Dorman, H.J. and Deans, S.G.**, *Antimicrobial agents from plants: antibacterial activity of plant volatile oils.* J Appl Microbiol, 2000. 88(2): p. 308-16.

245. **Lambert, R.J., Skandamis, P.N., Coote, P.J., and Nychas, G.J.**, *A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol.* J Appl Microbiol, 2001. 91(3): p. 453-62.

246. **Turgis, M., Han, J., Millette, M., Salmieri, S., Borsa, J., and Lacroix, M.**, *Effect of selected antimicrobial compounds on the radiosensitization of Salmonella Typhi in ground beef.* Lett Appl Microbiol, 2009. 48(6): p. 657-62.

247. **Ouhayoun, J.P.**, *Penetrating the plaque biofilm: impact of essential oil mouthwash.* J Clin Periodontol, 2003. 30 Suppl 5: p. 10-2.

248. **Carson, C.F., Mee, B.J., and Riley, T.V.**, *Mechanism of action of Melaleuca alternifolia (tea tree) oil on Staphylococcus aureus determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy.* Antimicrob Agents Chemother, 2002. 46(6): p. 1914-20.

249. **Patterson, G.H., Knobel, S.M., Sharif, W.D., Kain, S.R., and Piston, D.W.**, *Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy.* Biophys J, 1997. 73(5): p. 2782-90.

250. **Kneen, M., Farinas, J., Li, Y., and Verkman, A.S.**, *Green fluorescent protein as a noninvasive intracellular pH indicator.* Biophys J, 1998. 74(3): p. 1591-9.

251. **Iwami, Y., Kawarada, K., Kojima, I., Miyasawa, H., Kakuta, H., Mayanagi, H., and Takahashi, N.**, *Intracellular and extracellular pHs of Streptococcus mutans after addition of acids: loading and efflux of a fluorescent pH indicator in streptococcal cells.* Oral Microbiol Immunol, 2002. 17(4): p. 239-44.

252. **Oussalah, M., Caillet, S., Saucier, L., and Lacroix, M.**, *Antimicrobial effects of selected plant essential oils on the growth of a Pseudomonas putida strain isolated from meat.* Meat Sci, 2006. 73(2): p. 236-44.

253. **Ames, B.N., Lee, F.D., and Durston, W.E.**, *An improved bacterial test system for the detection and classification of mutagens and carcinogens.* Proc Natl Acad Sci U S A, 1973. 70(3): p. 782-6.

254. **Quillardet, P., Huisman, O., D'Ari, R., and Hofnung, M.**, *SOS chromotest, a direct assay of induction of an SOS function in Escherichia coli K-12 to measure genotoxicity.* Proc Natl Acad Sci U S A, 1982. 79(19): p. 5971-5.

255. **Evandri, M.G., Battinelli, L., Daniele, C., Mastrangelo, S., Bolle, P., and Mazzanti, G.**, *The antimutagenic activity of Lavandula angustifolia (lavender) essential oil in the bacterial reverse mutation assay.* Food Chem Toxicol, 2005. 43(9): p. 1381-7.

256. **Mezzoug, N., Elhadri, A., Dallouh, A., Amkiss, S., Skali, N.S., Abrini, J., Zhiri, A., Baudoux, D., Diallo, B., El Jaziri, M., and Idaomar, M.**, *Investigation of the mutagenic and antimutagenic effects of Origanum compactum essential oil and some of its constituents.* Mutat Res, 2007. 629(2): p. 100-10.

257. **Bassler, B.L.**, *How bacteria talk to each other: regulation of gene expression by quorum sensing.* Curr Opin Microbiol, 1999. 2(6): p. 582-7.

258. **Helander, I., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E., and Gorris, L.**, *Characterization of the action of selected essential oil components on Gram-negative bacteria.* J Agric Food Chem, 1998. 46: p. 3590-3595.

259. **Becerril, R., Gomez-Lus, R., Goni, P., Lopez, P., and Nerin, C.**, *Combination of analytical and microbiological techniques to study the antimicrobial activity of a new active food packaging containing cinnamon or oregano against E. coli and S. aureus.* Anal Bioanal Chem, 2007. 388(5-6): p. 1003-11.

260. **De Martino, L., De Feo, V., and Nazzaro, F.**, *Chemical composition and in vitro antimicrobial and mutagenic activities of seven Lamiaceae essential oils.* Molecules, 2009. 14(10): p. 4213-30.

261. **Camilli, A. and Bassler, B.L.**, *Bacterial small-molecule signaling pathways.* Science, 2006. 311(5764): p. 1113-6.

262. **Kjelleberg, S. and Molin, S.**, *Is there a role for quorum sensing signals in bacterial biofilms?* Curr Opin Microbiol, 2002. 5(3): p. 254-8.

263. **March, J.C. and Bentley, W.E.**, *Quorum sensing and bacterial cross-talk in biotechnology.* Curr Opin Biotechnol, 2004. 15(5): p. 495-502.

264. **Niu, C., Afre, S., and Gilbert, E.S.**, *Subinhibitory concentrations of cinnamaldehyde interfere with quorum sensing.* Lett Appl Microbiol, 2006. 43(5): p. 489-94.

265. **Khan, M.S., Zahin, M., Hasan, S., Husain, F.M., and Ahmad, I.**, *Inhibition of quorum sensing regulated bacterial functions by plant essential oils with special reference to clove oil.* Lett Appl Microbiol, 2009. 49(3): p. 354-60.

266. **Brackman, G., Cos, P., Maes, L., Nelis, H.J., and Coenye, T.**, *Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics in vitro and in vivo.* Antimicrob Agents Chemother, 2011. 55(6): p. 2655-61.

267. **Szabo, M.A., Varga, G.Z., Hohmann, J., Schelz, Z., Szegedi, E., Amaral, L., and Molnar, J.**, *Inhibition of quorum-sensing signals by essential oils.* Phytother Res, 2010. 24(5): p. 782-6.

268. **Lis-Balchin, M., Deans, S.G., and Eaglesham, E.**, *Relationship between bioactivity and chemical composition of commercial essential oils.* Flavour Frag. J, 1998. 13: p. 98-104.

269. **Fine, D.H.**, *Mouthrinses as adjuncts for plaque and gingivitis management. A status report for the American Journal of Dentistry.* Am J Dent, 1988. 1(6): p. 259-63.

270. **Administration, F.a.D.**, *Oral health care drug products for over-the-counter human use*, D.o.H.a.H. Services, Editor. 1982. p. 22712-22926.

271. **Sissons, C.H., Wong, L., and Cutress, T.W.**, *Inhibition by ethanol of the growth of biofilm and dispersed microcosm dental plaques.* Arch Oral Biol, 1996. 41(1): p. 27-34.

272. **Marchetti E, M.S., Marzo G, Civisca A, Di Pietro C, Marinelli G**, *Effect of an essential-oil containing mouthrinse on the reduction of plaque and interproximal inflammation in comparison with an ethanol solution.* Prev Assist Dent, 2009. 35(4): p. 123-127.

273. *Listerine Mentol Composition*. Available from: https://www.listerineprofessional.com/products/antiseptic-mouthwash-rinse/cool-mint#active-ingredients. Last access on 14/09/2017.

274. **Penugonda, B., Settembrini, L., Scherer, W., Hittelman, E., and Strassler, H.**, *Alcohol-containing mouthwashes: effect on composite hardness.* J Clin Dent, 1994. 5(2): p. 60-2.

275. **Smigel, K.**, *High-alcohol mouthwashes are under scrutiny.* J Natl Cancer Inst, 1991. 83(11): p. 751.

276. **Llewelyn, J.**, *Oral squamous cell carcinoma. Mouthwashes may increase risk.* BMJ, 1994. 308(6942): p. 1508.

277. **Bagan, J.V., Vera-Sempere, F., Marzal, C., Pellin-Carcelen, A., Marti-Bonmati, E., and Bagan, L.**, *Cytological changes in the oral mucosa after use of a mouth rinse with alcohol. A prospective double blind control study.* Med Oral Patol Oral Cir Bucal, 2012. 17(6): p. e956-61.

278. **Gandini, S., Negri, E., Boffetta, P., La Vecchia, C., and Boyle, P.**, *Mouthwash and oral cancer risk quantitative meta-analysis of epidemiologic studies.* Ann Agric Environ Med, 2012. 19(2): p. 173-80.

279. **Moazzez, R., Thompson, H., Palmer, R.M., Wilson, R.F., Proctor, G.B., and Wade, W.G.**, *Effect of rinsing with ethanol-containing mouthrinses on the production of salivary acetaldehyde.* Eur J Oral Sci, 2011. 119(6): p. 441-6.

280. **Lachenmeier, D.W.**, *Alcohol-containing mouthwash and oral cancer--can epidemiology prove the absence of risk?* Ann Agric Environ Med, 2012. 19(3): p. 609-10.

281. **McCullough, M.J. and Farah, C.S.**, *The role of alcohol in oral carcinogenesis with particular reference to alcohol-containing mouthwashes.* Aust Dent J, 2008. 53(4): p. 302-5.

282. **La Vecchia, C.**, *Mouthwash and oral cancer risk: an update.* Oral Oncol, 2009. 45(3): p. 198-200.

283. **Lang, N.P. and Brecx, M.**, *Chlorhexidine digluconate: an agent for chemical plaque control and prevention of gingival inflammation.* J Periodontal Res, 1986. 21: p. 74-89.

284. **Davies, G.E., Francis, J., Martin, A.R., Rose, F.L., and Swain, G.**, *1:6-Di-4'-chlorophenyldiguanidohexane (hibitane); laboratory investigation of a new antibacterial agent of high potency.* Br J Pharmacol Chemother, 1954. 9(2): p. 192-6.

285. **Ruppert, M. and Schlagenhauf, U.**, *La clorhexidina en Odontología. Aspectos generales. .* Quintessence (Ed. Española), 2005. 18: p. 12-23.

286. **Bascones, A. and Manso, F.**, *Clorhexidina en Odontoestomatología: Conceptos actuales y revisión de la literatura.* Avances en Odontoestomatología, 1994. 10: p. 685-708.

287. **Loe, H., Schiott, C.R., Karring, G., and Karring, T.**, *Two years oral use of chlorhexidine in man. I. General design and clinical effects.* J Periodontal Res, 1976. 11(3): p. 135-44.

288. **Manau Navarro, C. and Guasch Serra, S.**, *Métodos de control de la placa bacteriana*, in *Odontología preventiva y comunitaria: Principios, métodos y aplicaciones*, E. Cuenca Sala, C. Manau Navarro, and L. Serra Majem, Editors. 2003: Barcelona. p. 79.

289. **al-Tannir, M.A. and Goodman, H.S.**, *A review of chlorhexidine and its use in special populations.* Spec Care Dentist, 1994. 14(3): p. 116-22.

290. **Gilbert, P. and Moore, L.E.**, *Cationic antiseptics: diversity of action under a common epithet.* J Appl Microbiol, 2005. 99(4): p. 703-15.

291. **Arévalo, J., Arribas, J., Calbo, L., Hernández, M., Lizán, M., and Herruzco, R.**, *Guía del grupo de trabajo sobre desinfectantes y antisépticos. Revisión.* Medicina Preventiva, 1998. 4: p. 38-43.

292. **Albertos, J., Junquera, L., Albertos, M., Olay, S., and E, L.-A.**, *La clorhexidina. Perspectiva actual. .* Anales de Odontoestomatología, 1996. 5: p. 217-223.

293. **Franch, M., Pascual, A., and Santos, A.**, *Colutorios en periodoncia. Parte II.* Archivos de Odontoestomatología, 2005. 21: p. 471-487.

294. **Ellepola, A.N. and Samaranayake, L.P.**, *Adjunctive use of chlorhexidine in oral candidoses: a review.* Oral Dis, 2001. 7(1): p. 11-7.

295. **Gjermo, P., Bonesvoll, P., Hjeljord, L.G., and Rolla, G.**, *Influence of variation of pH of chlorhexidine mouth rinses on oral retention and plague-inhibiting effect.* Caries Res, 1975. 9(1): p. 74-82.

296. **Kohlbecker, G.**, *[Toxic impurities in chlorhexidine digluconate].* Dtsch Zahnarztl Z, 1989. 44(4): p. 273-6.

297. **Russell, A., Day, M., and 229238.**, *Antibacterial activity of chlorhexidine.* J Hosp Infect, 1993. 25: p. 229-238.

298. **Fardal, O. and Turnbull, R.S.**, *A review of the literature on use of chlorhexidine in dentistry.* J Am Dent Assoc, 1986. 112(6): p. 863-9.

299. **Veksler, A.E., Kayrouz, G.A., and Newman, M.G.**, *Reduction of salivary bacteria by pre-procedural rinses with chlorhexidine 0.12%.* J Periodontol, 1991. 62(11): p. 649-51.

300. **Beazley, V.C., Thrane, P., and Rolla, G.**, *Effect of mouthrinses with SnF2, LaCl3, NaF and chlorhexidine on the amount of lipoteichoic acid formed in plaque.* Scand J Dent Res, 1980. 88(3): p. 193-200.

301. **Katz, S.**, *The use of fluoride and chlorhexidine for the prevention of radiation caries.* J Am Dent Assoc, 1982. 104(2): p. 164-70.

302. **Ben-Yaakov, D., Friedman, M., Hirschfeld, Z., and Gedalia, I.**, *Fluoride enhancement of chlorhexidine uptake by hydroxyapatite and enamel powders.* J Oral Rehabil, 1984. 11(1): p. 65-70.

303. **Luoma, H.**, *Chlorhexidine solutions, gels and varnishes in caries prevention.* Proc Finn Dent Soc, 1992. 88(3-4): p. 147-53.

304. **Quirynen, M., Avontroodt, P., Peeters, W., Pauwels, M., Coucke, W., and van Steenberghe, D.**, *Effect of different chlorhexidine formulations in mouthrinses on de novo plaque formation.* J Clin Periodontol, 2001. 28(12): p. 1127-36.

305. **Herrera, D., Roldán, S., Santacruz, I., Santos, S., Masdevall, M., and Sanz, M.**, *Differences in antimicrobial activity of four commercial 0.12% chlorhexidine mouthrinse formulations: an in vitro contact test and salivary bacterial counts study.* J Clin Periodontol, 2003. 30(4): p. 307-14.

306. **Maris, P.**, *Modes of action of disinfectants.* Revue Scentifique et Technique (International Office of Epizootics), 1995. 14: p. 47-55.

307. **Maillard, J.Y.**, *Bacterial target sites for biocide action.* J Appl Microbiol, 2002. 92 Suppl: p. 16S-27S.

308. **Musteata, F.M. and Pawliszyn, J.**, *Assay of stability, free and total concentration of chlorhexidine in saliva by solid phase microextraction.* J Pharm Biomed Anal, 2005. 37(5): p. 1015-24.

309. **Rolla, G. and Melsen, B.**, *On the mechanism of the plaque inhibition by chlorhexidine.* J Dent Res, 1975. 54 Spec No B: p. B57-62.

310. **Hugo, W.B. and Longworth, A.R.**, *The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constituents, dehydrogenase activity and cell walls of Escherichia coli and Staphylococcus aureus.* J Pharm Pharmacol, 1966. 18(9): p. 569-78.

311. **Chawner, J.A. and Gilbert, P.**, *Interaction of the bisbiguanides chlorhexidine and alexidine with phospholipid vesicles: evidence for separate modes of action.* J Appl Bacteriol, 1989. 66(3): p. 253-8.

312. **Chawner, J.A. and Gilbert, P.**, *A comparative study of the bactericidal and growth inhibitory activities of the bisbiguanides alexidine and chlorhexidine.* J Appl Bacteriol, 1989. 66(3): p. 243-52.

313. **Bernimoulin, J.P.**, *Recent concepts in plaque formation.* J Clin Periodontol, 2003. 30 Suppl 5: p. 7-9.

314. **Leyes Borrajo, J.L., García, V.L., López, C.G., Rodríguez-Nuñez, I., García, F.M., and Gallas, T.M.**, *Efficacy of chlorhexidine mouthrinses with and without alcohol: a clinical study.* J Periodontol, 2002. 73(3): p. 317-21.

315. **Todkar, R., Sheikh, S., Byakod, G., and Muglikar, S.**, *Efficacy of chlorhexidine mouthrinses with and without alcohol - a clinical study.* Oral Health Prev Dent, 2012. 10(3): p. 291-6.

316. **Graziani, F., Gabriele, M., D'Aiuto, F., Suvan, J., Tonelli, M., and Cei, S.**, *Dental plaque, gingival inflammation and tooth -discolouration with different commercial -formulations of 0.2% chlorhexidine rinse: a double-blind randomised controlled clinical trial.* Oral Health Prev Dent, 2015. 13(2): p. 101-11.

317. **Ribeiro, L.G., Hashizume, L.N., and Maltz, M.**, *The effect of different formulations of chlorhexidine in reducing levels of mutans streptococci in the oral cavity: A systematic review of the literature.* J Dent, 2007. 35(5): p. 359-70.

318. **Roldán, S., Herrera, D., Santa-Cruz, I., O'Connor, A., González, I., and Sanz, M.**, *Comparative effects of different chlorhexidine mouth-rinse formulations on volatile sulphur compounds and salivary bacterial counts.* J Clin Periodontol, 2004. 31(12): p. 1128-34.

319. **Moran, J., Addy, M., and Newcombe, R.**, *A 4-day plaque regrowth study comparing an essential oil mouthrinse with a triclosan mouthrinse.* J Clin Periodontol, 1997. 24(9 Pt 1): p. 636-9.

320. **Riep, B.G., Bernimoulin, J.P., and Barnett, M.L.**, *Comparative antiplaque effectiveness of an essential oil and an amine fluoride/stannous fluoride mouthrinse.* J Clin Periodontol, 1999. 26(3): p. 164-8.

321. **Addy, M., Willis, L., and Moran, J.**, *Effect of toothpaste rinses compared with chlorhexidine on plaque formation during a 4-day period.* J Clin Periodontol, 1983. 10(1): p. 89-99.

322. **Kalaga, A., Addy, M., and Hunter, B.**, *Comparison of chlorhexidine delivery by mouthwash and spray on plaque accumulation.* J Periodontol, 1989. 60(3): p. 127-30.

323. **Addy, M., Jenkins, S., and Newcombe, R.**, *The effect of triclosan, stannous fluoride and chlorhexidine products on: (I) Plaque regrowth over a 4-day period.* J Clin Periodontol, 1990. 17(10): p. 693-7.

324. **Pizzo, G., La Cara, M., Licata, M.E., Pizzo, I., and D'Angelo, M.**, *The effects of an essential oil and an amine fluoride/stannous fluoride mouthrinse on supragingival plaque regrowth.* J Periodontol, 2008. 79(7): p. 1177-83.

325. **Singh, A., Daing, A., and Dixit, J.**, *The effect of herbal, essential oil and chlorhexidine mouthrinse on de novo plaque formation.* Int J Dent Hyg, 2013. 11(1): p. 48-52.

326. **Pan, P.H., Finnegan, M.B., Sturdivant, L., and Barnett, M.L.**, *Comparative antimicrobial activity of an essential oil and an amine fluoride/stannous fluoride mouthrinse in vitro.* J Clin Periodontol, 1999. 26(7): p. 474-6.

327. **Sliepen, I., Van Essche, M., Quirynen, M., and Teughels, W.**, *Effect of mouthrinses on Aggregatibacter actinomycetemcomitans biofilms in a hydrodynamic model.* Clin Oral Investig, 2010. 14(3): p. 241-50.

328. **Charles, C.H., Pan, P.C., Sturdivant, L., and Vincent, J.W.**, *In vivo antimicrobial activity of an essential oil-containing mouthrinse on interproximal plaque bacteria.* J Clin Dent, 2000. 11(4): p. 94-7.

329. **Fine, D.H., Furgang, D., Barnett, M.L., Drew, C., Steinberg, L., Charles, C.H., and Vincent, J.W.**, *Effect of an essential oil-containing antiseptic mouthrinse on plaque and salivary Streptococcus mutans levels.* J Clin Periodontol, 2000. 27(3): p. 157-61.

330. **Fine, D.H., Furgang, D., Lieb, R., Korik, I., Vincent, J.W., and Barnett, M.L.**, *Effects of sublethal exposure to an antiseptic mouthrinse on representative plaque bacteria.* J Clin Periodontol, 1996. 23(5): p. 444-51.

331. **Teles, R.P. and Teles, F.R.**, *Antimicrobial agents used in the control of periodontal biofilms: effective adjuncts to mechanical plaque control?* Braz Oral Res, 2009. 23 Suppl 1: p. 39-48.

332. **Lee, K.H., Kim, B.S., Keum, K.S., Yu, H.H., Kim, Y.H., Chang, B.S., Ra, J.Y., Moon, H.D., Seo, B.R., Choi, N.Y., and You, Y.O.**, *Essential oil of Curcuma longa inhibits Streptococcus mutans biofilm formation.* J Food Sci, 2011. 76(9): p. H226-30.

333. **Decker, E.M., Maier, G., Axmann, D., Brecx, M., and von Ohle, C.**, *Effect of xylitol/chlorhexidine versus xylitol or chlorhexidine as single rinses on initial biofilm formation of cariogenic streptococci.* Quintessence Int, 2008. 39(1): p. 17-22.

334. **Decker, E.M., von Ohle, C., Weiger, R., Wiech, I., and Brecx, M.**, *A synergistic chlorhexidine/chitosan combination for improved antiplaque strategies.* J Periodontal Res, 2005. 40(5): p. 373-7.

335. **Decker, E.M., Weiger, R., von Ohle, C., Wiech, I., and Brecx, M.**, *Susceptibility of planktonic versus attached Streptococcus sanguinis cells to chlorhexidine.* Clin Oral Investig, 2003. 7(2): p. 98-102.

336. **Deng, D.M., Buijs, M.J., and ten Cate, J.M.**, *The effects of substratum on the pH response of Streptococcus mutans biofilms and on the susceptibility to 0.2% chlorhexidine.* Eur J Oral Sci, 2004. 112(1): p. 42-7.

337. **Larsen, T. and Fiehn, N.E.**, *Development of a flow method for susceptibility testing of oral biofilms in vitro.* APMIS, 1995. 103(5): p. 339-44.

338. **Pratten, J., Smith, A.W., and Wilson, M.**, *Response of single species biofilms and microcosm dental plaques to pulsing with chlorhexidine.* J Antimicrob Chemother, 1998. 42(4): p. 453-9.

339. **Pratten, J., Wills, K., Barnett, P., and Wilson, M.**, *In vitro studies of the effect of antiseptic-containing mouthwashes on the formation and viability of Streptococcus sanguis biofilms.* J Appl Microbiol, 1998. 84(6): p. 1149-55.

340. **Thrower, Y., Pinney, R.J., and Wilson, M.**, *Susceptibilities of Actinobacillus actinomycetemcomitans biofilms to oral antiseptics.* J Med Microbiol, 1997. 46(5): p. 425-9.

341. **Wirthlin, M.R., Chen, P.K., and Hoover, C.I.**, *A laboratory model biofilm fermenter: design and initial trial on a single species biofilm.* J Periodontol, 2005. 76(9): p. 1443-9.

342. **Wilson, M., Patel, H., and Noar, J.H.**, *Effect of chlorhexidine on multi-species biofilms.* Curr Microbiol, 1998. 36(1): p. 13-8.

343. **Guggenheim, B., Giertsen, E., Schupbach, P., and Shapiro, S.**, *Validation of an in vitro biofilm model of supragingival plaque.* J Dent Res, 2001. 80(1): p. 363-70.

344. **Shapiro, S., Giertsen, E., and Guggenheim, B.**, *An in vitro oral biofilm model for comparing the efficacy of antimicrobial mouthrinses.* Caries Res, 2002. 36(2): p. 93-100.

345. **Jentsch, H., Mozaffari, E., and Jonas, L.**, *Scanning electron microscopy of growing dental plaque: a quantitative study with different mouth rinses.* Ultrastruct Pathol, 2013. 37(4): p. 233-40.

346. **Ulkur, F., Arun, T., and Ozdemir, F.**, *The effects of three different mouth rinses in a 4-day supragingival plaque regrowth study.* Eur J Dent, 2013. 7(3): p. 352-8.

347. **Marchetti, E., Mummolo, S., Di Mattia, J., Casalena, F., Di Martino, S., Mattei, A., and Marzo, G.**, *Efficacy of essential oil mouthwash with and without alcohol: a 3-day plaque accumulation model.* Trials, 2011. 12: p. 262.

348. **Marchetti, E., Casalena, F., Capestro, A., Tecco, S., Mattei, A., and Marzo, G.**, *Efficacy of two mouthwashes on 3-day supragingival plaque regrowth: a randomized crossover clinical trial.* Int J Dent Hyg, 2017. 15(1): p. 73-80.

349. **Marchetti, E., Tecco, S., Caterini, E., Casalena, F., Quinzi, V., Mattei, A., and Marzo, G.**, *Alcohol-free essential oils containing mouthrinse efficacy on three-day supragingival plaque regrowth: a randomized crossover clinical trial.* Trials, 2017. 18(1): p. 154.

350. **Pizzo, G., Compilato, D., Di Liberto, B., Pizzo, I., and Campisi, G.**, *Effects of two essential oil mouthrinses on 4-day supragingival plaque regrowth: a randomized cross-over study.* Am J Dent, 2013. 26(3): p. 156-60.

351. **Faul, F., Erdfelder, E., Lang, A.G., and Buchner, A.**, *G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences.* Behav Res Methods, 2007. 39(2): p. 175-91.

352. **CoreTeam.** *R: A language and environment for statistical computing.* 2016; Available from: http://www.R-project.org. Last access on 14/09/2017.

353. **Jentsch, H., Hombach, A., Beetke, E., and Jonas, L.**, *Quantitative transmission electron microscopic study of dental plaque--an in vivo study with different mouthrinses.* Ultrastruct Pathol, 2002. 26(5): p. 309-13.

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JUSTIFICATION AND OBJECTIVES

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Essential oils (EO) and chlorhexidine (CHX) are probably the most studied and popular oral antiseptics from all those commercially available. There are plenty of studies assessing their antibacterial activities and antiplaque effects on the oral biofilm. However, most of these investigations are of clinical character, in them analysing the effectiveness of antiseptics is performed by applying plaque indexes, obtaining very different results.

Other authors studied the oral biofilm microbiologically, by measuring colony forming units, describing the oral biofilm qualitatively by scanning electron microscope (SEM) or transmission electron microscope (TEM). The main drawback of these techniques is the impossibility of accurately analysing the characteristics of the oral biofilm due to the necessity of disrupting its delicate three-dimensional (3-D) architecture. Also, it should not be forgotten that the oral biofilm is hydrated and alive so a technique which keeps these characteristics would be very valuable. This method would permit the analysis of its structure, bacterial viability (BV) and the dynamic changes produced as time goes by.

The confocal laser scanning microscope (CLSM) together with dual live/dead staining techniques have demonstrated their efficacy and reliability at the analysis of *in situ* undisturbed oral biofilm. Besides, the appliances for the development of the oral biofilm *in situ* have improved their design and comfort, interfering as little as possible with the life of the volunteers [1].

There are few studies in the literature in which the effects of EO on *in situ* undisturbed oral biofilms have been evaluated by applying CLSM together with BV techniques [2, 3]. In addition, the appearance of alcohol-free EO formulations makes indispensable their evaluation in regard to the traditional solutions containing alcohol.

For all these reasons the objectives of the present Thesis are:

Objective 1. To evaluate the *in situ* immediate antibacterial effect and substantivity of a single traditional EO mouthwash on *de novo* undisturbed oral biofilm up to 7 hours after its application, using CLSM and a dual fluorescent staining solution.

Objective 2. To evaluate the *in situ* antiplaque effect of daily mouthwashes of traditional EO in the short term on *de novo* undisturbed oral biofilm, using CLSM and a dual fluorescent staining solution.

Objective 3: To compare the *in situ* antibacterial effects (immediate effect, substantivity and antiplaque effect) of the traditional EO with alcohol and a new formulation based on alcohol-free EO on *de novo* undisturbed oral biofilm, using CLSM and a dual fluorescent staining solution.

REFERENCES

1. **Prada-López, I., Quintas, V., Vilaboa, C., Suárez-Quintanilla, D., and Tomás, I.**, *Devices for in situ development of non-disturbed oral biofilm. a systematic review.* Front Microbiol, 2016. 7: p. 1055.

2. **Gosau, M., Hahnel, S., Schwarz, F., Gerlach, T., Reichert, T.E., and Burgers, R.**, *Effect of six different peri-implantitis disinfection methods on in vivo human oral biofilm.* Clin Oral Implants Res, 2010. 21(8): p. 866-72.

3. **Dong, W.L., Zhou, Y.H., Li, C.Z., Liu, H., Shang, S.H., and Pan, B.Q.**, *[Establishment and application of an intact natural model of human dental plaque biofilm].* Shanghai Kou Qiang Yi Xue, 2010. 19(2): p. 196-201.

OBJECTIVE 1

García-Caballero, L.; Quintas, V.; Prada-López, I.; Seoane, J.; Donos, N.; Tomás, I. Chlorhexidine substantivity on salivary flora and plaque-like biofilm: an *in situ* model. *PLoS One.* 2013 Dec 27; 8 (12):e83522. doi: 10.1371/journal.pone.0083522. eCollection 2013.

Quintas V.; Prada-López, I.; Prados-Frutos, J.C.; Tomás, I. *In situ* antimicrobial activity on oral biofilm: essential oils vs. 0.2 % chlorhexidine. *Clin Oral Investig*. 2015 Jan; 19 (1): 97-107. doi: 10.1007/s00784-014-1224-3. Epub 2014 Apr 1.

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OBJECTIVE 1. IMMEDIATE ANTIBACTERIAL EFFECT AND SUBSTANTIVITY OF A SINGLE MOUTHWASH OF TRADITIONAL ESSENTIAL OILS ON AN *IN SITU* MODEL OF UNDISTURBED ORAL BIOFILM: A RANDOMISED CLINICAL TRIAL

1.1. ABSTRACT

Introduction: The essential oils (EO) represent the oldest antiplaque and antigingivitis agent used clinically in dentistry. Studies performed *in situ* on the undisturbed dental plaque are considered of great value when assessing its efficacy on the oral biofilm.

Objectives: To evaluate the *in situ* antibacterial activity (immediate effect and substantivity) of a single mouthwash containing EO with alcohol (M-T-EO) on *de novo* undisturbed plaque-like biofilm (PL-biofilm) up to 7 hours after its application.

Material and Methods: An appliance was designed to hold six glass disks on the buccal sides of the lower teeth, allowing PL-biofilm growth. Fifteen healthy volunteers wore the device for 48 hours, and then they performed a M-T-EO. Disks were removed 30 seconds and 1, 3, 5, and 7 hours later. The same procedure was repeated in the same subjects after application of a single mouthwash of sterile water (M-WATER, negative control) and a single mouthwash of chlorhexidine M-0.2% (M-0.2% CHX, positive control). A 2-week washout period was established between the different rinsing protocols. After PL-biofilm vital staining, samples were analysed using a confocal laser scanning microscope (CLSM).

Results: At 30 seconds after M-T-EO, the mean level of bacterial viability (BV) was 1.18%, significantly lower than that detected in the basal sample (65.55%; $p<0.001$). After 7 hours, the antibacterial effect of M-T-EO was still patent with a 47.86% difference in BV compared to the basal sample ($p<0.001$). The M-T-EO obtained lower levels of BV at 1, 3 and 5 hours ($p<0.05$) but at 7 hours were as effective as 0.2% CHX (BV at 7 hours = 17.69 *vs.* 31.91%, $p>0.05$).

Conclusion: A single mouthwash of the traditional formulation of essential oils presented very high immediate antibacterial effect *in situ* and a substantivity which lasted for at least 7 hours after its application over a 48-hour PL-biofilm. These results were even better than those observed with 0.2% chlorhexidine under the same conditions.

Clinical relevance: A single mouthwash of the traditional formulation of essential oils is an effective measure against the *de novo* oral biofilm, representing a good alternative to chlorhexidine such as a preoperative rinse, in periodontal procedures or post-treatment applications.

1.2. INTRODUCTION

Nowadays, Listerine® is the most popular combination of essential oils (EO) [1] and represents the oldest antigingivitis and antiplaque agent used clinically in dentistry. It is considered safe and effective by the Committee of Experts on Oral Health of the FDA [2]. Listerine® contains a fixed combination of four EO as the active ingredients (thymol 0.064%, eucalyptol 0.092%, methyl-salicylate 0.060%, menthol 0.042%). In the traditional formulation of Listerine® (T-EO), these four EO are found solved in a solution containing from 21 to 26% of alcohol. EO kill microorganisms by disrupting their cell walls and inhibiting their enzymatic activity. They prevent bacterial aggregation, slow down bacterial multiplication, and extract endotoxins [3].

If it is assumed that biofilm bacteria may be 10-1000 times more resistant to antimicrobial agents than planktonic cells [4], a more reliable assessment of mouthwash efficacy may be better achieved with biofilm tests. Some studies have been performed on the antibacterial activity of EO on oral biofilm, both *in vitro* and *in situ*. The former, using artificial models, have helped us gain a better understanding of oral biofilms in spite of not being predictive of clinical activity derived from antiseptics [4-6]. These *in vitro* models involve a limited number of species, and they are conducted under conditions which do not reflect the physiological status of the oral ecosystem [7-9]. For these reasons, several authors have stated that results obtained from this type of study must be carefully interpreted [8, 10, 11].

With respect to *in situ* studies, they have greater value when establishing the antiseptic efficacy of several types of mouthwash, since their activity is tested under *in vivo* clinical conditions [12-15]. In these

studies, differences have been found between those performed on disturbed dental plaque [12, 13, 16] and those carried out on undisturbed dental plaque [14, 15]. In the first type, the plaque is analysed after being removed from the dental surface [12, 13, 16]. Due to this, it is not possible to assess either the original architecture of the biofilm or the penetration power of the antibacterial agent. Therefore, methodologies which permit biofilm formation under real clinical conditions are needed, so that plaque disturbance is not necessary for analysis. As a consequence, several types of removable devices, capable of holding multiple sorts of the substratum, have been designed. These devices produce a biofilm which is presumably similar to a dental plaque, generated under similar conditions and set over an artificial substratum; this has been called a plaque-like Biofilm (PL-biofilm).

Historically, various microscopy techniques have been used to visualise the PL-biofilm microstructure, including optic microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) [17]. Samples are distorted using these techniques, making correct analysis very difficult, especially in fluid-filled structures [18]. These problems have been eliminated or, at least, reduced with confocal laser scanning microscopy (CLSM). Its main advantage is to allow PL-biofilm analysis without altering its delicate structure [19]; also, this technique facilitates observation in “real” time. It also permits acquiring thin optical sections (0.5-2 µm) and examining the X-Z and X-Y relationships existing between the bacteria and their environment, significantly improving, at the same time, the lateral resolution [20].

In contrast to traditional microbial quantification methods, systems based on fluorescence have gained increasing importance, since they are

accepted as a simple, precise, reproducible, and highly sensitive procedure for quantifying adhered microorganisms [21]. Furthermore, although there is not a standard classification of the different bacterial states of viability, the staining capacity of the dyes present in the live/dead assays seems to match with the physiological condition of the bacteria, though there are still intermediate colours with “unknown” interpretation [21].

As a result, fluorescence staining has been incorporated to investigate biofilm structure and viability using a wide variety of dyes combinations, having been employed in PL-biofilm studies *in situ* [7, 22-25], due to their ability to stain live and dead bacteria selectively. The combination of SYTO 9 and propidium iodide (PI) has been proposed as a reliable alternative [26] to traditional blend of fluorescein diacetate (FDA) with ethidium bromide (EtBr), mainly because of the destructive properties of this combination and the toxicity and instability of the EtBr [26]. This dual staining method provided a visual demonstration of bactericidal activity as well as its quantification using computerised image analysis [12].

There are few studies in the literature in which the effects of T-EO on *in situ* undisturbed PL-biofilm have been measured by applying CLSM together with bacterial viability (BV) techniques [15]. The aim of the present study was to evaluate the *in situ* antibacterial activity (immediate bacterial effect and substantivity) of a single mouthwash of T-EO on undisturbed *de novo* PL-biofilm up to 7 hours after its application, using CLSM and a dual fluorescent staining solution.

1.3. MATERIAL AND METHODS

This investigation is a randomised, double blind, crossover study of the antibacterial activity of a single mouthwash of T-EO on an *in situ* model of PL-biofilm growth. The study meets the CONSORT checklist points and received the approval of the Clinical Research Ethics Committee of Galicia (number 2012/394).

1.3.1. SELECTION OF THE STUDY GROUP

To calculate an "a priori" sample size, the following statistical criteria were established: an effect size of 0.35, an alpha error of 0.05 and a statistical power of 80%. Assuming these criteria and the possible application of repeated measures ANOVA test, a sample size of 15 subjects was required. The sample size calculation was performed using the program G*Power 3.1.5 [27].

The participants were recruited among dental students at the Faculty of Medicine and Dentistry of Santiago de Compostela (Universidade de Santiago de Compostela, Spain), where volunteer enrollment was asked by responding to advertisements for the participation in a research study at the faculty hall. The study group was composed of 15 adult volunteers. All of these volunteers were revised by the same trained clinician to ensure they fulfilled all inclusion and exclusion criteria. The inclusion criteria were the following: being systemically healthy adult volunteers between 20 and 45 years old, who presented a good oral health status: a minimum of 24 permanent teeth with no evidence of gingivitis or periodontitis (Community Periodontal Index score = 0) [28] and an absence of untreated caries at the beginning of the study. The following exclusion criteria were applied: smoker or former smoker, the presence of dental prostheses or orthodontic

devices, antibiotic treatment or routine use of oral antiseptics in the previous 3 months, and the presence of any systemic disease that could alter the production or composition of saliva. Before the start of each study, a full mouth scaling with ultrasonic instruments and teeth polishing with rubber cup after dental disclosure was performed by the same trained clinician on all selected participants. Written informed consent was obtained from all participants in the study.

1.3.2. PRODUCTION OF THE INTRAORAL DEVICE OF OVERLAID DISK-HOLDING SPLINTS (IDODS)

The fabricated device was called the Intraoral Device of Overlaid Disk-holding Splints (IDODS, registered patent number: ES 2380252 B2).

In Figure 1.1 the IDODS manufacturing sequence is represented. Initially, a plaster model of the lower dental arch of each of the volunteers was needed. A first splint (inner sleeve) of ethylene-vinyl acetate copolymers (type Drufosoft, Dreve-Dentamid GmbH, Germany), that was soft, flexible and 1 mm thick, was made on each of these models. On this splint, six circular cavities of 2 mm in diameter were made; they were located in the vestibular area, between the canine-first premolar, second premolar-first molar and first molar-second molar in both hemi-arches.

On the inner splint, after trimming and perforations, six manufacturing or guide disks, 6 mm in diameter and 1 mm thick, each placed over an aperture. After that, on the plaster model with the splint and the set disks, a second splint (outer sleeve) of polyethylene terephthalate (type Biolon, CAS RN: 25038-59-9, from Dreve-Dentamid GmbH, Germany), that was

rigid and 1 mm thick, was prepared. Thus, the outer covered the inner splint, and the guide disks were housed in between them.

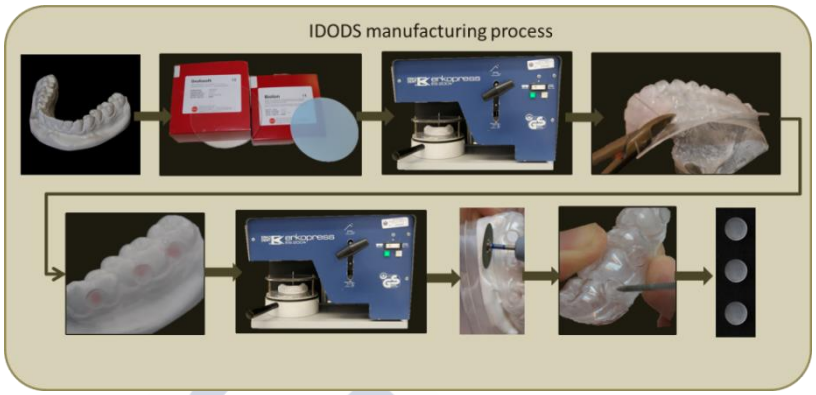


Figure 1.1. The sequence of fabrication of the Intraoral Device of Overlaid Disk-holding Splints (IDODS).

Subsequently, both splints were withdrawn from the plaster model to allow the removal of the guide disks and to clean up the excess of material fixing them. On the external splint, six circular cavities of 5 mm in diameter each were prepared. They were located in the same position as the perforations of the soft splint. Finally, the glass disks (on which the biofilm grows) were placed in their respective cavities, and both splints were joined with the application of heat to prevent any undesirable mobility during biofilm formation time.

The glass disks were lodged between the two splints, but with a surface of 5 mm exposed to buccal. This surface was protected from the action of the cheeks by an external splint frame surrounding the disk.

The IDODS design was modified during its use in this research. The most significant change was the ‘split-up design’ (Figure 1.2). With this enhancement, the splints were more comfortable for volunteers in their

normal life because their lower incisors were not covered. At the same time, this design facilitated the removal of the disks on the day of the sample analysis, preventing the unnecessary removal of the homologous splint.

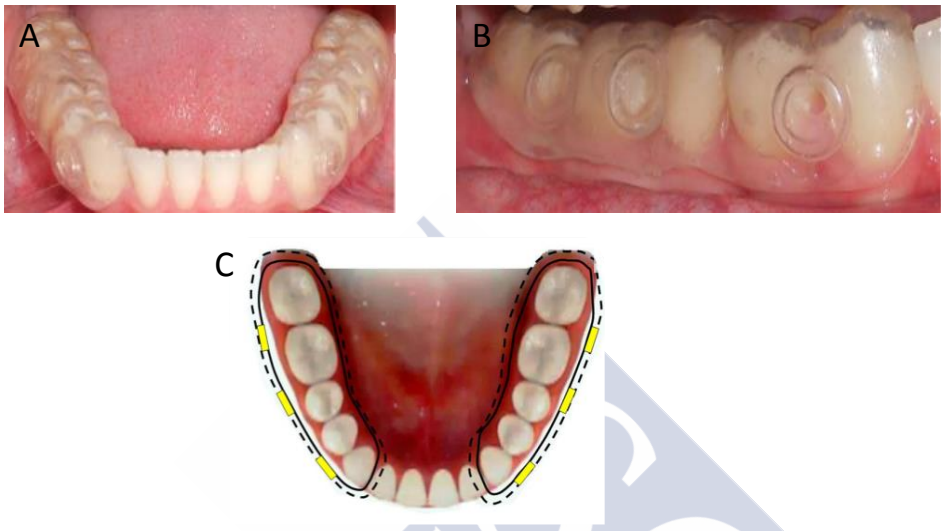


Figure 1.2. A) and B) Clinical images of the "split-mouth" design of the IDODS. C) Scheme of the "split-mouth" design of the IDODS. Note that the lower incisors are kept uncovered to improve aesthetics and comfort.

The splints were subjected to the following disinfection protocol before giving to the subjects: immersion in 3% NaCl solution for 1 minute in the ultrasonic cleaner, then 10 minutes in the ultrasonic cleaner in a 70% ethanol solution and finally, 10 minutes in distilled water. The splints were stored in distilled water for 24 hours the day before the start of the study with the objective of hydrating the materials [29].

The splints with the glass disks were worn by the volunteers for 48 hours to favour growth of the PL-biofilm, withdrawing it from the oral cavity only during meals (it was stored in an opaque container in humid conditions) and to perform oral hygiene procedures, using only mechanical removal of bacterial plaque with water without the use of any toothpaste or

mouthwash.

1.3.3. PROTOCOL OF THE STUDY (Figure 1.3)

After 48 hours, the glass disks were withdrawn one by one from the splint from each volunteer (from right to left in a distal-mesial direction) at baseline, 30 seconds, 1, 3, 5, and 7 hours after performing the following mouthwashes under supervision:

A) A single, 30-second mouthwash with 20 mL of sterile water (negative control) (M-WATER).

-OR

B) A single, 30-second mouthwash with 10 mL of 0.2% chlorhexidine (Oraldine Perio®, Johnson and Johnson, Madrid, Spain) (positive control) (M-0.2% CHX).

-OR

C) A single, 30-second mouthwash with 20 mL of essential oils in a hydroalcoholic solution (Listerine® Mentol™, Listerine®, Johnson & Johnson, Madrid, Spain) (M-T-EO).

On the day of the experiment, the volunteers were not allowed to eat or drink during the tests. A collection of the different PL-biofilm samples started at 11:50 AM (baseline sample) and finished at 7:00 PM (the final sample was obtained 7 hours after performing the mouthwash).

Using an internet-based balanced randomisation system [30], indicating the mouthwash each subject would use first, second and third, all volunteers performed the three mouthwashes, with a rest period of two

weeks between each test.

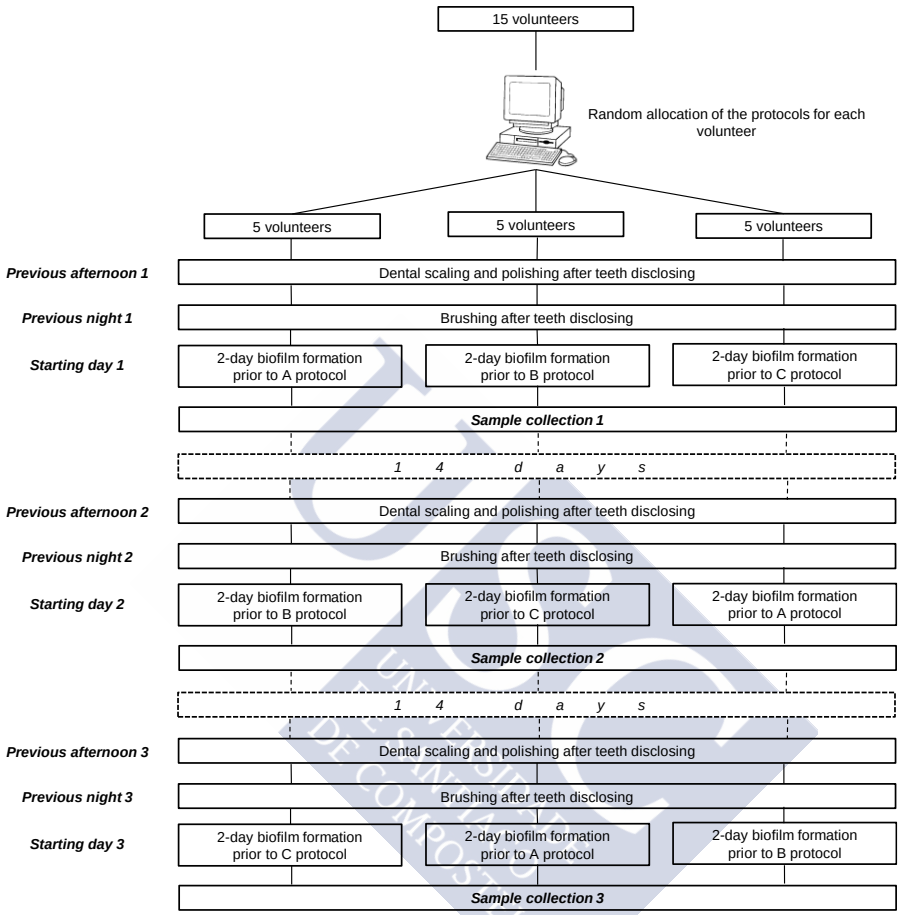


Figure 1.3. Protocol of the study.

1.3.4. PROCESSING OF THE SAMPLES OF THE PL-BIOFILM

The glass disks were withdrawn from the splint and were immediately submerged in 100 µL of fluorescence solution (Live/Dead® BacLight™ Kit, Molecular Probes Inc. Leiden, The Netherlands) and kept in a dark chamber at room temperature for 15 minutes. Microscopic observation was performed by a single investigator who was unaware of the study design, using a Leica TCS SP2 laser scanning spectral confocal microscope (Leica

Microsystems Heidelberg GmbH, Mannheim, Germany) with an HCX APOL 63x/0.9 water-immersion lens.

Four selected fields or XYZ series in the central part of each disk were evaluated. These fields were considered as representative of the whole sample after the observer's general examination. Fluorescence emission was determined in series of XY images in which each image corresponded to each of the Z positions (depth). The optical sections were scanned in 1 µm sections from the surface of the biofilm to its base, measuring the maximum thickness of the field and subsequently the mean thickness of the biofilm of the corresponding sample. The maximum thickness of biofilm field was defined as the distance between the substrate and the peaks of the highest cell clusters [31]. The maximum biofilm thickness of each field was divided into three zones or equivalent layers: the outer layer (layer 1), the middle layer (layer 2) and the inner layer (layer 3).

The capture of the data was done with the same settings in all cases. The spatial scan mode (XYZ) and the 1024x1024 pixels scan format resolution were used. The Argon-ion and Diode-pumped solid-state (DPSS) laser were used at a 13% and 78% of maximum intensity, respectively. The values for the pinhole, zoom and scan speed were 121.58 microns, 1 and 400Hz, respectively. The only values that were different depending on the sample were the offset (range between -1% to 1%) and photomultiplier (PMT) gain which was different for channel red and green. In general terms, these parameters were higher for green than for red (test and positive control), because there was more presence of red than green signal, being for the negative control the opposite. These values were always adjusted to get a good quality capture without background noise, avoiding excessive saturation of the brightest pixels of the image. As the

technician was blinded to the experiment, they were advised to make the adjustments always consistent with what was seeing by the objective of the microscope, obtaining an image which was the closest as possible to reality.

Quantification of BV in the series of XY images was determined using cytofluorographic analysis (Leica Confocal Software). In this analysis, the images of each fluorochrome were defined as “channels” (SYTO 9 occupies the green channel and PI the red channel). Square capture masks were used to measure the area occupied (μm^2) by the pixels in each channel, determining the total area occupied by the biofilm and the corresponding percentage of viability. The intensity ranges that were considered as positive signal were between 100 and 255. Determination of the mean percentage of BV in each field required sections with a minimum area of biofilm of 250 μm^2, and the mean percentage of BV of the biofilm was calculated for the corresponding sample and each biofilm layer.

1.3.5. STATISTICAL ANALYSIS

The results were analysed using the PASW® Statistics Base 18 package for Windows (IBM, Madrid, Spain). The data on thickness and BV in PL-biofilms are expressed as mean and standard deviation of the mean. All values from the quantitative variables analysed (thickness and BV) presented a normal distribution, which was determined using the Kolmogorov-Smirnov test. One-way ANOVA with repeated measures was used for intra-mouthwash comparisons using all the PL-biofilm samples. Two-way ANOVA with repeated measures was used for intra-mouthwash (differentiating between the three biofilm layers) and inter-mouthwash comparisons using all the PL-biofilm samples. Three-way ANOVA with repeated measures was used for inter-mouthwash (distinguishing between

the three biofilm layers) comparisons using all the PL-biofilm samples. Pairwise comparisons (with the Bonferroni adjustment) were used for the analysis of intra- and inter-mouthwash results (including differentiating between the three biofilm layers). Statistical significance was taken as a *p* value less than 0.05.

1.4. RESULTS

A total of 31 volunteers were evaluated to achieve the calculated sample size (n = 15). When this number of participants meeting the inclusion and exclusion criteria was reached, the enrollment process was ended. A total of 16 subjects were ineligible as they did not satisfy all of the inclusion criteria. All the 15 volunteers completed the rising protocols satisfactorily. In relation to demographic characteristics of the selected participants, eight were females and ten males with a mean age of 26.1 ± 2.9 years. No adverse or side effects were observed by investigators or reported by the volunteers after the completion of any of the rising protocols

1.4.1. INFLUENCE OF A SINGLE MOUTHWASH OF THE TRADITIONAL ESSENTIAL OILS SOLUTION ON THICKNESS OF THE PL-BIOFILM

The mean PL-biofilm thickness at baseline was 22.15 µm (range = 12 µm-28 µm). Significant differences were not found over time after M-T-EO with regard to the basal thickness. On the other hand, after M-0.2% CHX, lower values of PL-biofilm thickness were obtained in comparison to both the basal thickness and the M-T-EO thickness (Table 1.1).

Table 1.1. Measurement of PL-biofilm thickness, as well as intra-mouthwash and inter-mouthwash comparisons, before the different mouthwashes (baseline) and after (30 seconds, 1 hour, 3 hours, 5 hours, and 7 hours).

PL-biofilm THICKNESS Mean ± Standard Deviation (µm)						
	BASAL	30 SEC	1 H	3 H	5 H	7 H
M-WATER	19.32 ± 5.41	18.00 ± 2.56	22.25 ± 5.38	20.98 ± 5.12	23.87 ± 4.76	23.90 ± 3.92
M-0.2% CHX	23.43 ± 8.29	15.77 ± 1.87	13.47 ± 2.54	15.35 ± 2.93	17.52 ± 3.88	15.55 ± 2.32
M-T-EO	23.55 ± 4.66	20.88 ± 3.64	20.18 ± 4.26	20.95 ± 4.48	18.78 ± 4.40	21.57 ± 2.80

INTRA-MOUTHWASH ANALYSIS					
	BASAL *vs.* 30 SEC	BASAL *vs.* 1 H 30 SEC *vs.* 1 H	BASAL *vs.* 3 H 30 SEC *vs.* 3 H	BASAL *vs.* 5 H 30 SEC *vs.* 5 H	BASAL *vs.* 7 H 30 SEC *vs.* 7 H
M-WATER	----	---- *p*<0.05	---- ----	---- *p*<0.05	---- *p*<0.05
M-0.2% CHX	----	*p*<0.05 ----	*p*<0.05 ----	---- ----	*p*<0.05 ----
M-T-EO	----	---- ----	---- ----	---- ----	---- ----

INTER-MOUTHWASH ANALYSIS						
	BASAL	30 SEC	1 H	3 H	5 H	7 H
M-WATER *vs.* M-T-EO	----	----	----	----	*p*<0.05	----
M-WATER vs. M-0.2% CHX	----	----	*p*<0.001	*p*<0.05	*p*<0.05	*p*<0.001
M-0.2% CHX *vs.* M-T-EO	----	*p*<0.001	*p*<0.001	*p*<0.05	----	*p*<0.001

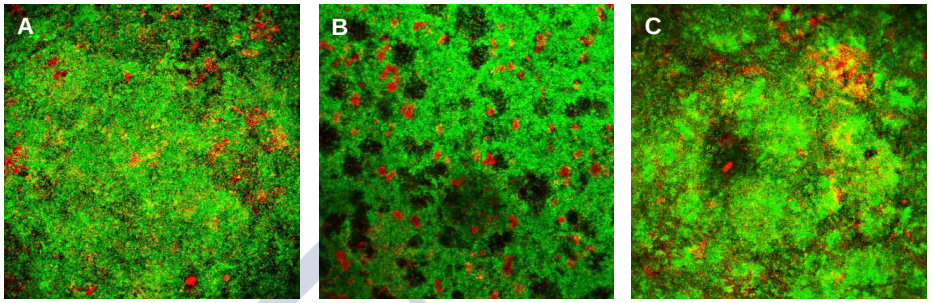
---- Not a statistically significant difference.

M-WATER = a single, 30-second mouthwash with 20 mL of sterile water; M-0.2% CHX = a single, 30-second mouthwash with 10 mL of 0.2% chlorhexidine; M-T-EO = a single, 30-second mouthwash with 20 mL of traditional essential oils.

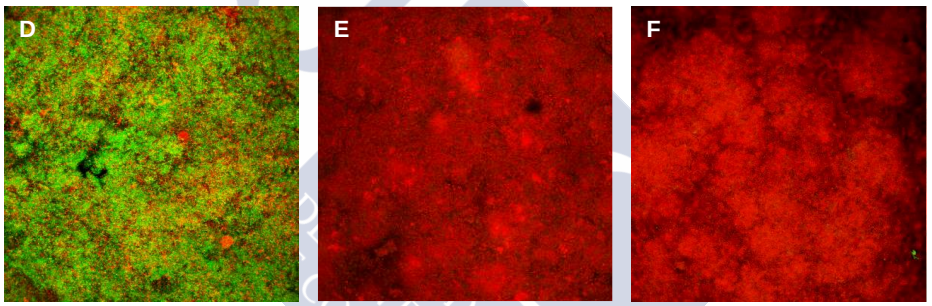
1.4.2. INFLUENCE OF A SINGLE MOUTHWASH OF THE TRADITIONAL ESSENTIAL OILS SOLUTION ON THE BACTERIAL VIABILITY OF THE PL-BIOFILM

The basal BV in PL-biofilm was 73.59% (44%-94%). The M-WATER

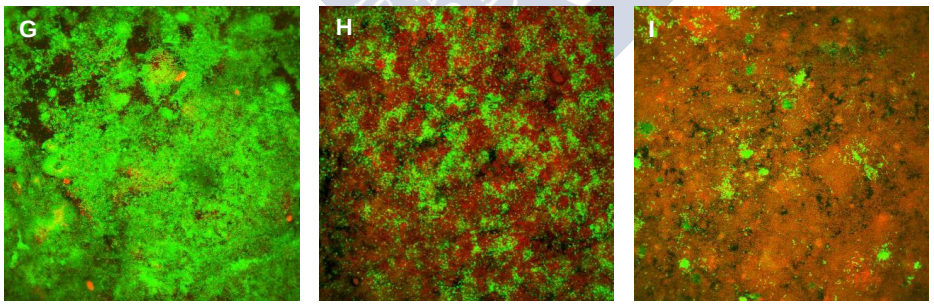
mouthwash did not have any significant effect on PL-biofilm viability compared to the basal level. The results after M-0.2% CHX and M-T-EO showed significant differences compared to their respective basal levels from 30 seconds after mouthwash use to 7 hours later (Figure 1.4).



Images A , B, C. Basal samples collected before different mouthwashes with M-WATER, M-0.2% CHX and M-T-EO, respectively.



Images D, E, F. 30 seconds after: M-WATER, M-0.2% CHX and M-T-EO, respectively.



Images G, H, I. 7 hours after: M-WATER, M-0.2% CHX and M-T-EO, respectively.

Figure 1.4. Representative images of the obtained bacterial viabilities at baseline, 30 seconds and 7 hours after a single mouthwash with sterile water (M-WATER), 0.2% chlorhexidine (M-0.2% CHX), and essential oils with alcohol (M-T-EO).

In comparison with the values obtained 30 seconds after M-0.2% CHX and M-T-EO, a significant recovery of the bacterial population was observed in the later PL-biofilm samples (after 3 hours and 5 hours, respectively). Comparing M-0.2% CHX and M-T-EO, the T-EO solution presented lower percentages of bacterial viability up to 7 hours after application, obtaining significant differences from 1 hour to 5 hours post-mouthwash (Figure 1.5).

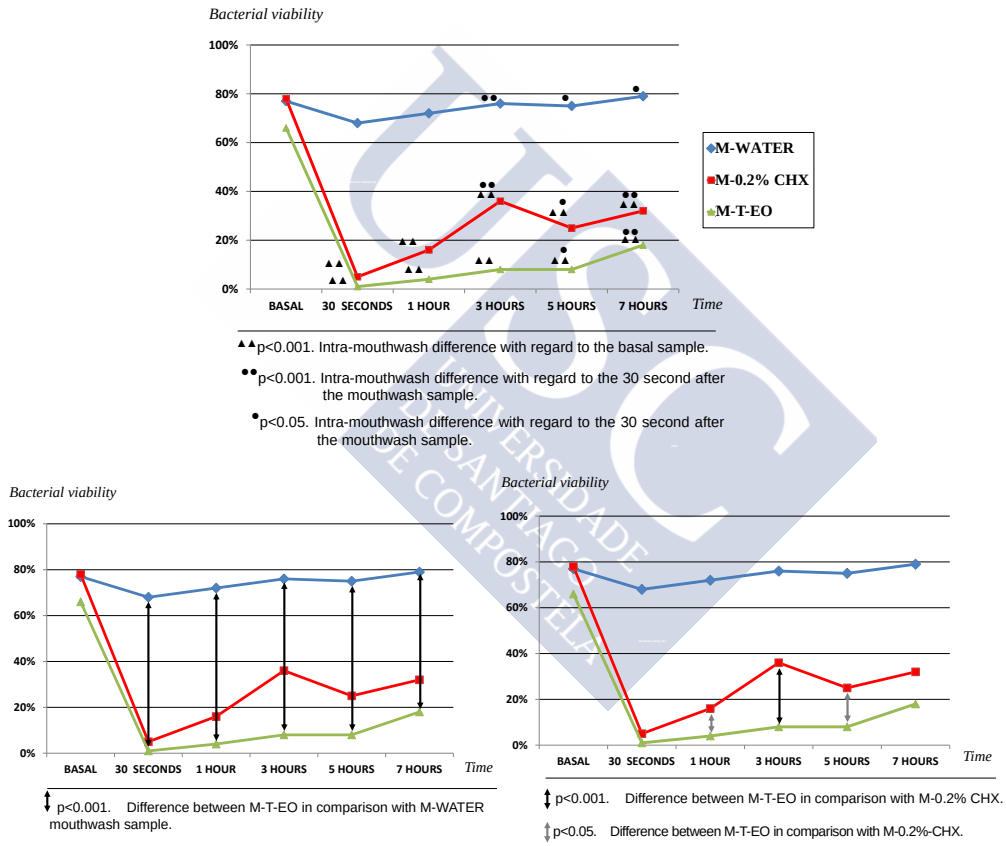


Figure 1.5. PL-biofilm bacterial viability percentage under basal conditions, and 30 seconds, 1, 3, 5, and 7 hours after a single water mouthwash (M-WATER), 0.2% chlorhexidine mouthwash (M-0.2% CHX) or traditional essential oil mouthwash (M-T-EO).

Differentiating between the three biofilm layers, the prevalence of viable bacteria under basal conditions was higher in the outer layers concerning

deeper layers, reaching statistical significance in the majority of comparisons (Table 1.2).

Compared with M-WATER, the prevalence of viable bacteria was significantly lower in the three biofilm layers in all the biofilm samples taken after M-T-EO ($p<0.05$ in all comparisons). Compared with M-0.2% CHX, the prevalence of viable bacteria was significantly lower in the middle and inner layers from 1 hour after mouthwash use to 7 hours later in all the biofilm samples taken after M-T-EO ($p<0.05$ and $p<0.001$ for all comparisons) (Table 1.2).

Table 1.2. Mean percentages of bacterial viability in PL-biofilm under basal conditions and in the samples collected at 30 seconds and 1, 3, 5, and 7 hours after a single mouthwash of sterile water, 0.2% chlorhexidine and traditional essential oils, differentiating between the three biofilm layers, as well as intra-mouthwash and inter-mouthwash comparisons.

PL-biofilm BACTERIAL VIABILITY DIVIDED INTO LAYERS Mean ± Standard Deviation (%)						
	BASAL	30 SEC	1 H	3 H	5 H	7 H
M-WATER						
Layer 1	85.36 ± 6.56	85.27 ± 13.10	88.30 ± 9.63	90.82 ± 8.91	88.98 ± 8.51	90.93 ± 5.94
Layer 2	79.81 ± 7.28	73.08 ± 15.13	78.44 ± 16.56	84.44 ± 10.32	81.34 ± 12.81	85.20 ± 7.06
Layer 3	66.83 ± 27.28	45.80 ± 33.35	49.39 ± 29.79	56.73 ± 31.46	55.43 ± 24.91	61.96 ± 24.00
M-0.2% CHX						
Layer 1	79.95 ± 6.22	5.21 ± 6.20	15.14 ± 15.44	35.42 ± 15.53	21.71 ± 19.75	27.04 ± 22.64
Layer 2	82.22 ± 7.83	5.06 ± 6.43	16.54 ± 15.61	36.71 ± 16.43	24.83 ± 20.62	28.66 ± 20.76
Layer 3	71.81 ± 17.44	4.98 ± 5.04	15.17 ± 10.21	35.16 ± 14.83	27.45 ± 13.46	40.04 ± 20.38
M-T-EO						
Layer 1	78.65 ± 10.65	1.67 ± 1.59	5.04 ± 4.33	13.72 ± 14.01	15.83 ± 13.16	37.46 ± 19.09
Layer 2	68.96 ± 16.51	1.14 ± 1.18	2.94 ± 3.29	5.95 ± 10.19	5.36 ± 5.32	12.46 ± 9.90
Layer 3	49.05 ± 35.95	0.72 ± 0.56	4.06 ± 6.83	5.00 ± 8.21	3.61 ± 7.01	3.16 ± 2.87

INTRA-MOUTHWASH ANALYSIS						
	BASAL	30 SEC	1 H	3 H	5 H	7 H
M-WATER						
Layer 1 *vs.* Layer 2	$p<0.05$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
Layer 1 *vs.* Layer 3	----	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
Layer 2 *vs.* Layer 3	----	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
M-CHX 0.2%						

Layer 1 *vs.* Layer 2	----	----	----	----	-----	----
Layer 1 *vs.* Layer 3	----	----	----	----	----	*p*<0.05
Layer 2 *vs.* Layer 3	*p*<0.05	----	----	----	----	*p*<0.05
M-EO						
Layer 1 *vs.* Layer 1	*p*<0.05	*p*<0.05	*p*<0.05	*p*<0.001	*p*<0.05	*p*<0.001
Layer 2 *vs.* Layer 2	*p*<0.05	----	----	----	*p*<0.05	*p*<0.001
Layer 3 *vs.* Layer 3	*p*<0.05	----	-----	----	----	*p*<0.05

INTER-MOUTHWASH ANALYSIS						
	BASAL	30 SEC	1 H	3 H	5 H	7 H
M-WATER *vs.* M-T-EO						
Layer 1 *vs.* Layer 1	----	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001
Layer 2 *vs.* Layer 2	----	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001
Layer 3 *vs.* Layer 3	----	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001
M-WATER *vs.* M-0.2% CHX						
Layer 1 *vs.* Layer 1	----	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001
Layer 2 *vs.* Layer 2	----	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001
Layer 3 *vs.* Layer 3	----	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001
M-0.2% CHX *vs.* M-T-EO						
Layer 1 *vs.* Layer 1	----	----	----	*p*<0.05	----	----
Layer 2 *vs.* Layer 2	*p*<0.05	----	*p*<0.05	*p*<0.001	*p*<0.05	----
Layer 3 *vs.* Layer 3	----	*p*<0.05	*p*<0.05	*p*<0.001	*p*<0.001	*p*<0.001

---- Not a statistically significant difference.

M-WATER = a single, 30-second mouthwash with 20 mL of sterile water; M-0.2% CHX = A single, 30-second mouthwash with 10 mL of 0.2% chlorhexidine; M-T-EO = a single, 30-second mouthwash with 20 mL of traditional essential oils.

1.5. DISCUSSION

1.5.1. METHODOLOGICAL APPROACH

There has been marked inter-individual variability detected regarding the characteristics of PL-biofilm [8, 19, 25, 32]. In the present study, a sample group of 15 individuals was selected. This sample group is bigger than in similar studies in which the number of volunteers ranged from 3 to 10 [14, 15, 19, 33, 34]. With regard to the type of removable appliance used to collect the supragingival dental plaque, devices such as the Leeds *in situ* device [9, 18, 35, 36], bilateral mandibular stents [32, 37, 38], and different types of individualised acrylic splints [7, 8, 10, 19, 22, 24] have been

previously described. In the present series, two individualised splints formed of two sheets were designed for each volunteer. The splint was composed of an internal vinyl sheet to which three disks were attached, with an external polyethylene terephtalate sheet that was fenestrated to permit contact between the vestibular surface of the disks and the saliva while protecting the surface from the action of the cheeks and tongue. The disks were positioned on each hemiarch and inserted towards the interdental area between two adjacent teeth to imitate an approximate PL-biofilm, which is only minimally influenced by the forces of the oral soft tissues. This particular design ensured that the biofilm was not touched or disturbed during removal or repositioning of the appliance [39]. In contrast to previous designs [7, 8, 19], where the splints referred to a complete model, the partial model of IDODS represents a new approach in the way of making a better *in situ* biofilm model. This redesign was more comfortable for the participants when talking and wearing the splints because their incisors were not covered. At the same time, the extraction of the disks was easier, because it was not necessary to remove the whole inferior arch splint but only the hemiarch corresponding to the analysis, keeping the other undisturbed.

A number of solid substrates of different characteristics have been used in published studies on PL-biofilm, including human enamel [18, 23, 24, 38], bovine enamel [10, 22, 34], bovine dentine [25, 34], hydroxyapatite [14], polished glass [7, 8, 19, 23], and titanium [15]. The roughness of the surface of the substrate and its free energy are considered to be important factors for the *in situ* growth of PL-biofilm [8]. However, Netuschil et al. [23] found no major differences in the thickness of 2-day PL-biofilm using enamel or glass disks. In addition, a recent study conducted by our group compared the BV, thickness and composition of the PL-biofilm formed on

the surface of artificial disks of enamel, hydroxyapatite and glass with the biofilm formed on the natural teeth surface. No differences were found between the artificial substrates and with regard the natural tooth; in fact, results were more conditioned by the interindividual differences (unpublished data). On the other hand, due to the known autofluorescence of enamel, using glass is recommended to avoid any visual disturbance, mainly in the deepest layers of the biofilm [23]. By these findings, in the present series, glass disks were used for *in situ* growth of the 2-day PL-biofilm.

Lately, there have been some discussions about the suitability of Live/Dead® BacLight™ in the study of natural multispecies bacterial environments [40]. It has been stated that this particular solution has the general tendency to artificially increase the levels of non-vital bacteria [40]. Conversely, our group has previously demonstrated that epifluorescence microscopy and CLSM combined with SYTO 9/PI dual stain is a useful method for quantifying the antibacterial activity of CHX on salivary flora and PL-biofilms in real time [41-43]. In addition, in another previous experiment [44], the authors found a correlation between the immediate effect and the plate cultures. However, this correlation was lost as time passed, with the antibacterial effect of CHX being overestimated by the plate cultures [44]. These results suggested that either the BV is underestimated with plate culture or overestimated with fluorescence techniques, which is in contrast to what has been recently stated [40].

Previous *in situ* studies on substantivity of antiseptics have been conducted by our group in such a way that at least six different samples can be obtained from the same volunteer in a period of 7 hours. In these samples, a very diverse BV was observed, depending on the time at which

the samples were obtained [45]. In these cases, is the fluorescence solution artificially raising the dead bacteria, coincidentally, just after the mouthwash? What happens in the baseline? Is the fluorescence solution doing its job well at this time and then, suddenly, increases the number of dead bacteria? Or is it due to the antibacterial action of mouthwash? Why is there a general tendency towards a significant increase in BV in all subjects after an antiseptic mouthwash with fluorescence techniques as time passes (which is logical from a physiological point of view), although the traditional plate culture techniques cannot appreciate this fact? Hannig et al. [46] considered that live/dead staining methods were reliable when analysing antimicrobial agents activity. Nevertheless, they continued to ask the question about "how dead is dead?" due to several stages of viability which have been discussed and described in the literature (viable and culturable, viable but non-culturable, dormant, non-viable and pre-lytic, and avital dead bacteria). The exact differentiation of these stages is still one of the greatest challenges in modern microbiology [47]. Also, and in contrast to the affirmations of Netuschil et al. [40], Tawakoli et al. [26] demonstrated that the BacLight® system was a reliable alternative when assessing BV in a 120-minute old natural dental biofilm, in which there are already present several types of bacteria. The Live/dead® BacLight™ fluorescence assay stains the bacteria in red or green depending on the permeability of their membrane; given that the tested antiseptics act mostly at this cellular element, this vital staining method is suitable for this type of study. Furthermore, Tawakoli et al. [26] affirmed that it was not possible to compare accurately the viability assessed with fluorescence staining solutions with traditional plaque cultures. It is well known the wide limitations of this last method (among others, only 50% of the oral bacteria are culturable), which emphasises the necessity of the using of viability assays [48].

Accordingly, the fact that these results are coherent with clinical reality and have been obtained from studies with a crossover design makes that this technique can be considered valid for the evaluation of the viability in oral biofilms.

1.5.2. INFLUENCE OF A SINGLE MOUTHWASH OF THE TRADITIONAL ESSENTIAL OILS SOLUTION ON THE THICKNESS AND BACTERIAL VIABILITY OF THE PL-BIOFILM

Studies, which have analysed *in situ* PL-biofilm, have emphasised the considerable variation detected in biofilm thickness between individuals [8, 24, 33]; this was also observed in the present study (mean value of PL-biofilm thickness after two days was 22.10 µm, ranging from 12 µm to 28 µm). These results indicated that, in agreement with previous statements, "*the height of the oral biofilms formed depended on the plaque-forming rate of the individual donors*" [24]. Our mean value of PL-biofilm was consistent with that obtained by Dong et al. [14] under similar conditions, which was 27.55 µm.

In the present series, the BV of 2-day PL-biofilm in baseline was approximately 73%. These results are consistent with previous studies, which reported mean BV of PL-biofilm between 60% and 77% over 2- and 3-day periods [7, 19, 49]. Consequently, viable microorganisms were located on and embedded in dead layers, which may be responsible for further plaque growth [23].

In some series, large inter-individual differences were found among the subjects in their PL-biofilm viability distribution [25], so no general pattern for the BV distribution could be described [25, 49]; in the present study, the PL-biofilm viability ranged from 44% to 90%. However, it has been

suggested that a relatively constant ecological environment exists in each volunteer, which obviously leads to a microbial identity pattern [19]. In this sense, Arweiler et al. [19] detected significant variation in the BV values in a 2-day PL-biofilm for the different biofilm layers, identifying three viability patterns. In this study, despite the high degree of variability detected in the BV distribution, a viability pattern could be determined, which was based on a low viability percentage observed in the layers nearest to the substrate, increasing in outer layers. This finding confirms the importance of the dead cellular material in the initial states of PL-biofilm development. This will mainly help its growth, and this material will protect it from antibacterial agents in the oral cavity [23, 24].

In this objective, the results with M-0.2% CHX were taken as a positive control, since our research group has deeply analysed and discussed 0.2% CHX antimicrobial activity on *in situ* PL-biofilm in previous publications [45]. Although some papers related to T-EO antimicrobial activity on biofilm *in vitro* have been published, [3-6, 50, 51], studies on the *in situ* effects of a single application of TEO on PL-biofilm applying CLSM and BV techniques are very scarce. From them, in two cases, the antiseptic treatment was practised *ex vivo*, which means that no mouthwash was done [14, 15]. In the other three [12, 13, 16], the studied plaque was thoroughly disturbed by the recollection method (paper points or curettes). Furthermore, only one article has been published in which the authors compared T-EO and CHX antimicrobial activity after a single application (application *ex vivo*), and they only measured the immediate antimicrobial effect [15]. Consequently, the present results have been compared with those obtained in other studies that applied different methodologies.

- *Thickness reduction*

As the results of the current series show, a single EO application is not effective in reducing PL-biofilm thickness. These findings coincide with those previously described by Dong et al. [14] in a 48-hour biofilm, who did not find significant differences in biofilm thickness with regard to the basal sample after applying M-T-EO. These findings are also consistent with an *in vitro* study conducted by Sliepen et al. [3], who observed that T-EO caused nearly no changes in biofilm structure, thickness, and surface coverage. Concerning M-0.2% CHX, statistically significant differences in the PL-biofilm thickness were found compared to M-T-EO at 30 seconds, 1, 3, and 7 hours after mouthwash use, which could suggest a possible antiplaque effect of 0.2% CHX after a single antiseptic application.

Moreover, the group of Charles [16] suggested that the clinical effectiveness of a single application of T-EO against plaque and gingivitis may be attributable to its bactericidal and penetration into the dental plaque.

- *Immediate antibacterial effect and substantivity*

With a similar basal viability between the three mouthwashes (mean of 73.6%), a clear and immediate post-mouthwash effect was detected after T-EO application. This immediate activity was very high if compared to that obtained in a similar study reported by Gosau et al. [15], since in the previous study, BV after M-T-EO was around 20%, while in the present series, the viability was 1% 30 seconds after M-T-EO. The methodological differences should be noted; in the previous case, *ex vivo* disk immersion was performed, while in the present series, the volunteers themselves carried out the mouthwash, providing an *in situ* antiseptic application. This

is evidently a more reliable approximation of the clinical situation. Therefore, it was found that moving the EO solution around the mouth and the force imposed by the cheeks when projecting it onto the PL-biofilm is probably of prime importance for reducing biofilm viability [42]. In the same way, this form of application can also favour the penetration capacity of the antiseptic compared to when the rinse was not actively applied. As expected, the negative control (M-WATER) had no antibacterial activity compared to M-T-EO. In comparison with the positive control (M-0.2% CHX), a single mouthwash of T-EO was more effective, consistent with previous results obtained by other groups [3, 15].

In the present series, T-EO antimicrobial activity was detectable until 7 hours after mouthwash application, when the reduction in viability was still 61%. In this study, it was also appreciated that a M-T-EO was effective for maintaining low BV levels in PL-biofilm. As shown in Figure 1.4, there were no statistically significant differences in BV until 5 hours compared to the 30 seconds post-mouthwash sample, which indicates potent antibacterial activity at high levels until that moment. Up to now, there have been two studies on the substantivity of T-EO in oral biofilm. The first found 21.3% viability at 30 minutes post-mouthwash [12], while the other, conducted by Fine [13], showed a viability reduction of 88% after 12 hours post-mouthwash. Both studies used a disturbed dental plaque *in vivo* model, so these results are not entirely comparable.

Regarding the positive control (M-0.2% CHX), there were significant differences from 1 hour to 5 hours post-mouthwash, and the M-T-EO was more effective at maintaining low values of BV. These findings are not described in the available literature due to the lack of studies measuring the substantivity of T-EO in oral biofilm in comparison with CHX.

Another interesting aspect of this study is the higher penetration capacity into the PL-biofilm of T-EO compared to 0.2% CHX. Statistically significant differences were found in BV reduction in layer 3 (the deepest one) between both antiseptics, starting from the immediate and 1-hour samples, but more so when time passed, and until 7 hours post-mouthwash. This finding indicates that the penetration capacity of a single application of T-EO is greater than that shown by a single application of 0.2% CHX [42]. As Pan et al. [12] previously described, these results confirm the ability of the T-EO mouthwash to penetrate plaque and exert its bactericidal activity *in situ* rapidly. Apart from that, the M-T-EO maintains its antimicrobial activity in the deepest layers (closest to the theoretical tooth surface) for a longer time.

There have been other *in situ* studies demonstrating the efficacy of a single mouthwash of these antiseptics in other oral ecosystems, such as the saliva. It has been shown that a M-T-EO or a M-0.2% CHX can significantly reduce the levels of recoverable salivary bacteria compared to negative control mouthwashes for periods of 5-7 hours [44, 52]. Both studies showed higher BV than those values found in PL-biofilm of the present series, so a longer substantivity period of both antiseptics, T-EO and 0.2% CHX) was detected in the 2-day PL-biofilm.

In addition, in another publication from our research group, Tomás et al. [53] compared the efficacy of an M-0.2% CHX at reducing the BV in saliva and 2-day PL-biofilms, both nocturnally and diurnally. With M-0.2% CHX-diurnal, the frequency of viable bacteria in saliva was significantly higher than in the PL-biofilm at 8, 10, and 12 hours after mouthwash. After M-0.2% CHX-nocturnal, the percentage of viable bacteria in saliva was significantly lower than in the PL-biofilm at 8 hours and higher than in the

PL-biofilm at 12 hours after mouthwash. These findings corroborate the more active physiologic dynamics of the salivary flora and the possible reservoir function associated with the structure of undisturbed *de novo* PL-biofilm.

Subsequently, other authors demonstrated that performing daily mouthwashes with a T-EO solution has a considerable antiplaque effect [54, 55]. However, two recent literature reviews [56, 57] concluded that daily T-EO mouthwash use has a lower antiplaque effect than using 0.2% CHX, although the gingival inflammation levels were quite similar in the long- and short-term. Based on these findings, it would be very interesting to analyse the antiplaque effect associated with continuous T-EO use in a 4-day *in situ* "undisturbed" PL-biofilm model as the next step in this research.

1.6. CONCLUSION

A single mouthwash of the traditional formulation of essential oils presented very high immediate antibacterial effect *in situ* and a substantivity which lasted for at least 7 hours after its application over *de novo* undisturbed plaque-like biofilm. These results were even better than those observed with 0.2% chlorhexidine under the same conditions.

Consequently, a single mouthwash of essential oils containing alcohol is an effective measure against the *de novo* oral biofilm, representing a good alternative to chlorhexidine such as a preoperative rinse, in periodontal procedures or post-treatment applications.

1.7. REFERENCES

1. **Vlachojannis, C., Winsauer, H., and Chrubasik, S.**, *Effectiveness and safety of a mouthwash containing essential oil ingredients.* Phytother Res: PTR, 2013. 27(5): p. 685-91.

2. **Administration, F.a.D.**, *Oral Health Care Drug Products for Over-the-Counter Human Use; Antigingivitis/ Antiplaque Drug Products; Establishment of a Monograph; Proposed Rules Part III.* , F.a.D.A.D.o.H.a.H. Services, Editor. 2003. p. part. 356.

3. **Sliepen, I., Van Essche, M., Quirynen, M., and Teughels, W.**, *Effect of mouthrinses on Aggregatibacter actinomycetemcomitans biofilms in a hydrodynamic model.* Clin Oral Investig, 2010. 14(3): p. 241-50.

4. **Fine, D.H., Furgang, D., and Barnett, M.L.**, *Comparative antimicrobial activities of antiseptic mouthrinses against isogenic planktonic and biofilm forms of Actinobacillus actinomycetemcomitans.* J Clin Periodontol, 2001. 28(7): p. 697-700.

5. **Filoche, S.K., Coleman, M.J., Angker, L., and Sissons, C.H.**, *A fluorescence assay to determine the viable biomass of microcosm dental plaque biofilms.* J Microbiol Methods, 2007. 69(3): p. 489-96.

6. **Pan, P.C., Harper, S., Ricci-Nittel, D., Lux, R., and Shi, W.**, *In-vitro evidence for efficacy of antimicrobial mouthrinses.* J Dent, 2010. 38 Suppl 1: p. S16-20.

7. **Auschill, T.M., Hein, N., Hellwig, E., Follo, M., Sculean, A., and Arweiler, N.B.**, *Effect of two antimicrobial agents on early in situ biofilm formation.* J Clin Periodontol, 2005. 32(2): p. 147-52.

8. **Auschill, T.M., Hellwig, E., Sculean, A., Hein, N., and Arweiler, N.B.**, *Impact of the intraoral location on the rate of biofilm growth.* Clin Oral Investig, 2004. 8(2): p. 97-101.

9. **Watson, P.S., Pontefract, H.A., Devine, D.A., Shore, R.C., Nattress, B.R., Kirkham, J., and Robinson, C.**, *Penetration of fluoride into natural plaque biofilms.* J Dent Res, 2005. 84(5): p. 451-5.

10. **Al-Ahmad, A., Wunder, A., Auschill, T.M., Follo, M., Braun, G., Hellwig, E., and Arweiler, N.B.**, *The in vivo dynamics of Streptococcus spp., Actinomyces naeslundii, Fusobacterium nucleatum and Veillonella spp. in dental plaque biofilm as analysed by five-colour multiplex fluorescence in situ hybridization.* J Med Microbiol, 2007. 56(Pt 5): p. 681-7.

11. **Hannig, C. and Hannig, M.**, *The oral cavity--a key system to understand substratum-dependent bioadhesion on solid surfaces in man.* Clin Oral Investig, 2009. 13(2): p. 123-39.

12. **Pan, P., Barnett, M.L., Coelho, J., Brogdon, C., and Finnegan, M.B.**, *Determination of the in situ bactericidal activity of an essential oil mouthrinse using a vital stain method.* J Clin Periodontol, 2000. 27(4): p. 256-61.

13. **Fine, D.H., Furgang, D., Sinatra, K., Charles, C., McGuire, A., and Kumar, L.D.**, *In vivo antimicrobial effectiveness of an essential oil-containing mouth rinse 12 h after a single use and 14 days' use.* J Clin Periodontol, 2005. 32(4): p. 335-40.

14. **Dong, W.L., Zhou, Y.H., Li, C.Z., Liu, H., Shang, S.H., and Pan, B.Q.**, *[Establishment and application of an intact natural model of human dental plaque biofilm].* Shanghai Kou Qiang Yi Xue, 2010. 19(2): p. 196-201.

15. **Gosau, M., Hahnel, S., Schwarz, F., Gerlach, T., Reichert, T.E., and Burgers, R.**, *Effect of six different peri-implantitis disinfection methods on in vivo human oral biofilm.* Clin Oral Implants Res, 2010. 21(8): p. 866-72.

16. **Charles, C.H., Pan, P.C., Sturdivant, L., and Vincent, J.W.**, *In vivo antimicrobial activity of an essential oil-containing mouthrinse on interproximal plaque bacteria.* J Clin Dent, 2000. 11(4): p. 94-7.

17. **Hannig, C., Hannig, M., Rehmer, O., Braun, G., Hellwig, E., and Al-Ahmad, A.**, *Fluorescence microscopic visualization and quantification of initial bacterial colonization on enamel in situ.* Arch Oral Biol, 2007. 52(11): p. 1048-56.

18. **Wood, S.R., Kirkham, J., Marsh, P.D., Shore, R.C., Nattress, B., and Robinson, C.**, *Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy.* J Dent Res, 2000. 79(1): p. 21-7.

19. **Arweiler, N.B., Hellwig, E., Sculean, A., Hein, N., and Auschill, T.M.**, *Individual vitality pattern of in situ dental biofilms at different locations in the oral cavity.* Caries Res, 2004. 38(5): p. 442-7.

20. **Wright, S.J. and Wright, D.J.**, *Introduction to confocal microscopy.* Methods Cell Biol, 2002. 70: p. 1-85.

21. **Hahnel, S., Rosentritt, M., Burgers, R., and Handel, G.**, *Surface properties and in vitro Streptococcus mutans adhesion to dental resin polymers.* J Mater Sci Mater Med, 2008. 19(7): p. 2619-27.

22. **Arweiler, N.B., Lenz, R., Sculean, A., Al-Ahmad, A., Hellwig, E., and Auschill, T.M.**, *Effect of food preservatives on in situ biofilm formation.* Clin Oral Investig, 2008. 12(3): p. 203-8.

23. **Netuschil, L., Reich, E., Unteregger, G., Sculean, A., and Brecx, M.**, *A pilot study of confocal laser scanning microscopy for the assessment of undisturbed dental plaque vitality and topography.* Arch Oral Biol, 1998. 43(4): p. 277-85.

24. **Auschill, T.M., Arweiler, N.B., Netuschil, L., Brecx, M., Reich, E., and Sculean, A.**, *Spatial distribution of vital and dead microorganisms in dental biofilms.* Arch Oral Biol, 2001. 46(5): p. 471-6.

25. **Zaura-Arite, E., van Marle, J., and ten Cate, J.M.**, *Conofocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm.* J Dent Res, 2001. 80(5): p. 1436-40.

26. **Tawakoli, P.N., Al-Ahmad, A., Hoth-Hannig, W., Hannig, M., and Hannig, C.**, *Comparison of different live/dead stainings for detection and quantification of adherent microorganisms in the initial oral biofilm.* Clin Oral Investig, 2013. 17(3): p. 841-50.

27. **Faul, F., Erdfelder, E., Lang, A.G., and Buchner, A.**, *G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences.* Behav Res Methods, 2007. 39(2): p. 175-91.

28. **World Health Organization**, *Oral health surveys: basic methods*. 5th ed. 2013, France. 125.

29. **Deimling, D., Breschi, L., Hoth-Hannig, W., Ruggeri, A., Hannig, C., Nekrashevych, Y., Prati, C., and Hannig, M.**, *Electron microscopic detection of salivary alpha-amylase in the pellicle formed in situ.* Eur J Oral Sci, 2004. 112(6): p. 503-9.

30. **Dallal, G.E.**; Available from: www.randomization.com. Last access on 03/09/2017

31. **Roberts, S.K., Bass, C., Brading, M., Lappin-Scott, H., and Stoodley, P.**, *Biofilm formation and structure; What's new?*, in *Dental plaque revisited-oral biofilms in health and disease*, W.M. Newman HN, Editor. 1999, BioLine: Cardiff. p. 1-36.

32. **Diaz, P.I., Chalmers, N.I., Rickard, A.H., Kong, C., Milburn, C.L., Palmer, R.J., Jr., and Kolenbrander, P.E.**, *Molecular characterization of subject-specific oral microflora during initial colonization of enamel.* Appl Environ Microbiol, 2006. 72(4): p. 2837-48.

33. **Dige, I., Nyengaard, J.R., Kilian, M., and Nyvad, B.**, *Application of stereological principles for quantification of bacteria in intact dental biofilms.* Oral Microbiol Immunol, 2009. 24(1): p. 69-75.

34. **Jung, D.J., Al-Ahmad, A., Follo, M., Spitzmuller, B., Hoth-Hannig, W., Hannig, M., and Hannig, C.**, *Visualization of initial bacterial colonization on dentine and enamel in situ.* J Microbiol Methods, 2010. 81(2): p. 166-74.

35. **Wood, S., Nattress, B., Kirkham, J., Shore, R., Brookes, S., Griffiths, J., and Robinson, C.**, *An in vitro study of the use of photodynamic therapy for the treatment of natural oral plaque biofilms formed in vivo.* J Photochem Photobiol B, 1999. 50(1): p. 1-7.

36. **Robinson, C., Strafford, S., Rees, G., Brookes, S.J., Kirkham, J., Shore, R.C., Watson, P.S., and Wood, S.**, *Plaque biofilms: the effect of chemical environment on natural human plaque biofilm architecture.* Arch Oral Biol, 2006. 51(11): p. 1006-14.

37. **Palmer, R.J., Jr., Gordon, S.M., Cisar, J.O., and Kolenbrander, P.E.**, *Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque.* J Bacteriol, 2003. 185(11): p. 3400-9.

38. **Chalmers, N.I., Palmer, R.J., Jr., Du-Thumm, L., Sullivan, R., Shi, W., and Kolenbrander, P.E.**, *Use of quantum dot luminescent probes to achieve single-cell resolution of human oral bacteria in biofilms.* Appl Environ Microbiol, 2007. 73(2): p. 630-6.

39. **Tomás, I., Cousido, M.C., García-Caballero, L., Rubido, S., Limeres, J., and Diz, P.**, *Substantivity of a single chlorhexidine mouthwash on salivary flora: influence of intrinsic and extrinsic factors.* J Dent, 2010. 38(7): p. 541-6.

40. **Netuschil, L., Auschill, T.M., Sculean, A., and Arweiler, N.B.**, *Confusion over live/dead stainings for the detection of vital microorganisms in oral biofilms - which stain is suitable?* BMC Oral Health, 2014. 14: p. 2.

41. **Cousido, M.C., Tomás Carmona, I., García-Caballero, L., Limeres, J., Álvarez, M., and Diz, P.**, *In vivo substantivity of 0.12% and 0.2% chlorhexidine mouthrinses on salivary bacteria.* Clin Oral Investig, 2010. 14(4): p. 397-402.

42. **Prada-López, I., Quintas, V., Casares-De-Cal, M.A., Suarez-Quintanilla, J.A., Suárez-Quintanilla, D., and Tomás, I.**, *Ex vivo vs. in vivo antibacterial activity of two antiseptics on oral biofilm.* Front Microbiol, 2015. 6: p. 655.

43. **Prada-López, I., Quintas, V., Donos, N., Suárez-Quintanilla, D., and Tomás, I.**, *Characteristics of in situ oral biofilm after 2 and 4 days of evolution.* Quintessence Int, 2015. 46(4): p. 287-298.

44. **Tomás, I., García-Caballero, L., Cousido, M.C., Limeres, J., Álvarez, M., and Diz Dios, P.**, *Evaluation of chlorhexidine substantivity on salivary flora by epifluorescence microscopy.* Oral Dis, 2009. 15(6): p. 428-33.

45. **García-Caballero, L., Quintas, V., Prada-López, I., Seoane, J., Donos, N., and Tomás, I.**, *Chlorhexidine substantivity on salivary flora and plaque-like biofilm: an in situ model.* PLoS One, 2013. 8(12): p. e83522.

46. **Hannig, C., Follo, M., Hellwig, E., and Al-Ahmad, A.**, *Visualization of adherent micro-organisms using different techniques.* J Med Microbiol, 2010. 59(Pt 1): p. 1-7.

47. **Decker, E.M.**, *The ability of direct fluorescence-based, two-colour assays to detect different physiological states of oral streptococci.* Lett Appl Microbiol, 2001. 33(3): p. 188-92.

48. **Aas, J.A., Paster, B.J., Stokes, L.N., Olsen, I., and Dewhirst, F.E.**, *Defining the normal bacterial flora of the oral cavity.* J Clin Microbiol, 2005. 43(11): p. 5721-32.

49. **von Ohle, C., Gieseke, A., Nistico, L., Decker, E.M., DeBeer, D., and Stoodley, P.**, *Real-time microsensor measurement of local metabolic activities in ex vivo dental biofilms exposed to sucrose and treated with chlorhexidine.* Appl Environ Microbiol, 2010. 76(7): p. 2326-34.

50. **Pan, P.H., Finnegan, M.B., Sturdivant, L., and Barnett, M.L.**, *Comparative antimicrobial activity of an essential oil and an amine fluoride/stannous fluoride mouthrinse in vitro.* J Clin Periodontol, 1999. 26(7): p. 474-6.

51. **Fine, D.H., Furgang, D., Barnett, M.L., Drew, C., Steinberg, L., Charles, C.H., and Vincent, J.W.**, *Effect of an essential oil-containing antiseptic mouthrinse on plaque and salivary Streptococcus mutans levels.* J Clin Periodontol, 2000. 27(3): p. 157-61.

52. **Jenkins, S., Addy, M., Wade, W., and Newcombe, R.G.**, *The magnitude and duration of the effects of some mouthrinse products on salivary bacterial counts.* J Clin Periodontol, 1994. 21(6): p. 397-401.

53. **Tomás, I., García-Caballero, L., López-Álvar, E., Suárez, M., Diz, P., and Seoane, J.**, *In situ chlorhexidine substantivity on saliva and plaque-like biofilm: influence of circadian rhythm.* J Periodontol, 2013. 84(11): p. 1662-72.

54. **Stoeken, J.E., Paraskevas, S., and van der Weijden, G.A.**, *The long-term effect of a mouthrinse containing essential oils on dental plaque and gingivitis: a systematic review.* J Periodontol, 2007. 78(7): p. 1218-28.

55. **Cortelli, S.C., Cortelli, J.R., Wu, M.M., Simmons, K., and Charles, C.A.**, *Comparative antiplaque and antigingivitis efficacy of a multipurpose essential oil-containing mouthrinse and a cetylpyridinium chloride-containing mouthrinse: A 6-month randomized clinical trial.* Quintessence Int, 2012. 43(7): p. e82-94.

56. **Van Leeuwen, M.P., Slot, D.E., and Van der Weijden, G.A.**, *Essential oils compared to chlorhexidine with respect to plaque and parameters of gingival inflammation: a systematic review.* J Periodontol, 2011. 82(2): p. 174-94.

57. **Neely, A.L.**, *Essential oil mouthwash (EOMW) may be equivalent to chlorhexidine (CHX) for long-term control of gingival inflammation but CHX appears to perform better than EOMW in plaque control.* J Evid Based Dent Pract, 2012. 12(3 Suppl): p. 69-72.

OBJECTIVE 2

Quintas, V.; Prada-López, I.; Donos, N.; Suárez-Quintanilla, D.; Tomás, I. Antiplaque effect of essential oils and 0.2% chlorhexidine on an in situ model of oral biofilm growth: a randomised clinical trial. *PLoS One*. 2015 Feb 17; 10 (2): e0117177. doi: 10.1371/journal.pone.0117177. eCollection 2015.

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OBJECTIVE 2. ANTIPLAQUE EFFECT OF A 4-DAY MOUTHWASH PROTOCOL OF TRADITIONAL ESSENTIAL OILS ON AN *IN SITU* MODEL OF UNDISTURBED ORAL BIOFILM: A RANDOMISED CLINICAL TRIAL

2.1. ABSTRACT

Introduction: Models of 4-day of oral biofilm maturation have been found useful for the assessment of the antiplaque effect of oral antiseptics.

Objective: To evaluate the *in situ* antiplaque effect after 4 days of using of two commercial antimicrobial agents in the short term on undisturbed plaque-like biofilm (PL-biofilm).

Material and Methods: An observer-masked**,** crossover randomised clinical trial on 15 oral and systemically healthy volunteers between 20-30 years, who were randomly and sequentially allocated in the same group and performed three interventions in different randomised sequences. The participants wore an intraoral appliance in three different rinsing periods doing mouthwashes twice a day (1/0/1) during the 4-day period with essential oils containing alcohol (4D-T-EO), 0.2% chlorhexidine (4D-0.2% CHX; positive control) or sterile water (4D-WATER; negative control). At the end of each 4-day mouthwash period, samples were removed from the appliance. Posteriorly, after viability staining, samples were analysed using a confocal laser scanning microscope (CLSM), analysing its thickness, covering grade (CG) and bacterial viability (BV) by the PL-biofilm. This Clinical Trial was registered at clinicaltrials.gov with the number NCT02124655. URL: https://clinicaltrials.gov/ct2/show/NCT02124655.

Results: The 4D-T-EO and the 4D-0.2% CHX were significantly more effective than the 4D-WATER at reducing thickness, CG and BV of the PL-biofilm. No significant differences were found between the 4D-T-EO and 4D-0.2% CHX at reducing the BV (14.7% *vs*. 13.2%). However, the 4D-0.2% CHX showed more reduction than the 4D-T-EO in thickness (6.5 µm *vs.* 10.0 µm; $p<0.05$) and CG by the PL-biofilm (20.0% *vs.* 54.3%; $p<0.001$).

Conclusion: The essential oils containing alcohol and 0.2% chlorhexidine showed a very high antiplaque effect. Although the 0.2% chlorhexidine showed better results with regard to reducing the thickness and covering grade by the biofilm, both antiseptics showed a high and similar antibacterial activity.

Clinical relevance: Daily essential oils containing alcohol or 0.2% chlorhexidine mouthwashes are effective in reducing dental plaque formation in the short term. Although 0.2% chlorhexidine continues to be the *Gold Standard* regarding the antiplaque effect, traditional essential oils are a reliable alternative.

2.2. INTRODUCTION

The accumulation of oral biofilm in the gingival margin is widely recognised to be the primary aetiological factor in the development of chronic gingivitis [1, 2]. Based on this association, the current treatment of gingivitis is focused on biofilm disruption, which will normally include mechanical processes, both professionally and at home. However, for patients, it is not easy to achieve a proper level of plaque control. The efficient plaque control techniques are very time consuming and require an individual motivation and skills for their optimum use [3]. It was at this point where mouthwashes become important because they include diverse types of antimicrobial agents to complement the results of mechanical oral hygiene measures [4].

The chlorhexidine (CHX) is considered the *Gold Standard* of oral antiseptics; nevertheless, it has not been recommended for extended periods of time due to its well-known secondary effects [5]. All of these inconveniences have limited its acceptability among dental professionals and users; on the other hand, these drawbacks have motivated the interest of the researchers in other alternative antiplaque agents [6]. Mouthwashes containing essential oils and alcohol (T-EO) in their formulation have received a lot of attention. Their antiplaque activity has been demonstrated in numerous clinical studies, in which they were used in conjunction with mechanical oral hygiene measures [7, 8].

Taking into account the fact that bacteria present in a biofilm can be from 10-1000 times more tolerant to antimicrobial agents than those in planktonic phase [9], a more reliable evaluation of the efficacy of an antiseptic agent present in mouthwash could be done using biofilm models.

Numerous studies have been carried out regarding the activity of EO and CHX on the oral biofilm both *in vitro* and *in situ*. The latter is more valuable when establishing the antiseptic efficacy of mouthwashes, due to the fact that their antibacterial activity is tested under *in vivo* clinical conditions [10].

To achieve a better understanding of the clinical effects that these agents produce in the interior of the oral biofilm, it is necessary to apply a methodology in which the biofilm grows directly in the interior of the oral cavity, but its three-dimensional (3-D) structure is not distorted by manipulation [11, 12]. Most *in situ* studies on undisturbed dental plaque, the authors did not use natural teeth; instead, they used disks made of different materials that were introduced in the mouth for a variable period of time, during which they were exposed to the intraoral conditions of an individual. The plaque generated in the artificial substrate is the already named plaque-like biofilm (PL-biofilm) (Objective 1).

The study of the antiplaque effect of an antimicrobial agent can be performed in long and short term clinical studies. Among the latter, 4-day models have particular importance. This model can be described as an established method for assessing the inhibitory activity against dental plaque that mouthwashes have, *per se,* and determines the relative effectiveness of the different formulations [13, 14]. Thereby, they have been widely used by various research groups to study various antiplaque agents that are commonly employed in the oral cavity [13-17].

Confocal laser scanning microscopy (CLSM), despite having poorer resolution than transmission electron microscopy (TEM) or scanning electron microscopy (SEM) [18], has eliminated or considerably reduced the distortion produced by preparation of the samples. The main advantage of

CLSM is that it permits the analysis of biofilm *in situ*, without altering its delicate structure, keeping it hydrated with no need for fixation or drying [19, 20]. CLSM also facilitates the observation of biofilms *in situ* and in "real" time, with all of the benefits of most sophisticated image analysis [21].

Systems based on the identification of bacterial viability (BV) by fluorescence have become increasingly important since they are accepted as a simple, accurate, reproducible and highly sensitive method for the quantification of attached microorganisms [21]. Consequently, numerous authors have examined biofilms with the help of CLSM and fluorescence staining, incorporating both to analyse their structure [11, 22], such as the spatial distribution of the vital and non-vital bacteria [23-25]. Various combinations of dyes have been used in the literature [10, 11, 26-28]. The combination of SYTO 9 and propidium iodide (PI) has been proposed as a reliable alternative [29] to traditional blend of fluorescein diacetate (FDA) with ethidium bromide (EtBr), mainly because of the destructive properties of this combination and the toxicity and instability of the EtBr [29]. This staining method provides a visual demonstration of bactericidal activity as well as its quantification using computerised image analysis [30].

The aim of this study was to evaluate the *in situ* antiplaque effect of two antimicrobial agents in the short term with the following analysis on undisturbed biofilm with CLSM combined with dual fluorescent staining solution.

2.3. MATERIAL AND METHODS

This investigation is a randomised, observer-masked, crossover study of the antiplaque efficacy of two available formulas based on EO containing alcohol (Listerine® Mentol™, Listerine® Johnson & Johnson, Madrid, Spain) and 0.2% CHX (Oraldine® Perio™ Johnson & Johnson, Madrid, Spain) on an *in situ* model of PL-biofilm growth. The study meets the CONSORT checklist points and received the approval of the Clinical Research Ethics Committee of Galicia (number 2012/393). The trial was registered in ClinicalTrials.gov with the ID number NCT02124655. URL: https://clinicaltrials.gov/ct2/show/NCT02124655.

2.3.1. SELECTION OF THE STUDY GROUP

To calculate an "a priori" sample size, the following statistical criteria were established: an effect size of 0.35, an alpha error of 0.05 and a statistical power of 80%. Assuming these criteria and the possible application of repeated measures ANOVA test, a sample size of 15 subjects was required (Figure 2.1). The sample size calculation was performed using the program G*Power 3.1.5. [31].

The participants were collected among dental students at the Faculty of Medicine and Dentistry of Santiago de Compostela (Universidade de Santiago de Compostela), where volunteer enrollment was asked by advertisements asking for the participation in a research study at the Faculty hall. All of these volunteers were revised by the same trained clinician to ensure they fulfilled all inclusion and exclusion criteria. The selected volunteers met the same inclusion and exclusion criteria reported in the previous objective (Objective 1). The inclusion criteria were the following: being systemically healthy adult volunteers between 20 and 45 years old,

who presented a good oral health status (a minimum of 24 permanent teeth with no evidence of gingivitis or periodontitis -Community Periodontal Index score = 0- [32] and an absence of untreated caries at the beginning of the study). The following exclusion criteria were applied: smoker or former smoker, the presence of dental prostheses or orthodontic devices, antibiotic treatment or routine use of oral antiseptics in the previous 3 months, and the presence of any systemic disease that could alter the production or composition of saliva.

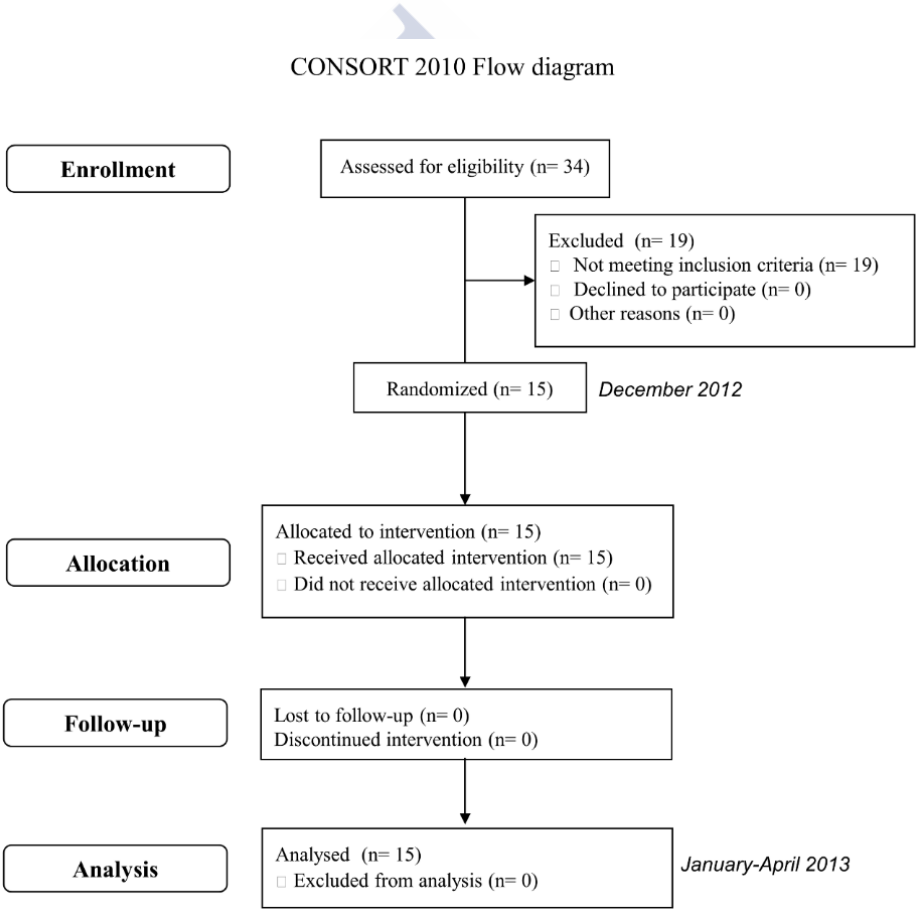

Figure 2.1. Flow diagram of the study with enrollment, allocation, follow-up and analysis of participants.

Before the start of each study, a full mouth scaling with ultrasonics and teeth polishing with rubber cup after dental disclosing was performed by the same calibrated clinician on all selected participants. Written informed consent was obtained from all participants in the study.

2.3.2. PRODUCTION OF THE INTRAORAL DEVICE OF OVERLAID DISK-HOLDING SPLINTS (IDODS)

Two Intraoral Device of Overlaid Disk-holding Splints (IDODS) were designed for each participant. These IDODS were produced applying the same protocol given in the previous objective (Objective 1). Each IDODS was capable of holding six glass disks (6 mm in diameter, 1 mm thickness) and these were polished at 800 grit.

2.3.3. PROTOCOL OF THE STUDY (Figure 2.2)

During the 4 days (96 hours) of duration of each mouthwash protocol of the present study, each volunteer wore the splints with the glass disks, withdrawing them from the oral cavity only during meals and to perform oral hygiene procedures, using only the mechanical removal of bacterial plaque with water, without the use of any toothpaste or mouthwash. They were advised to do just three meals per day avoiding eating or drinking in-between. The only drink permitted during meals was still water. While eating and brushing their teeth, the splints were stored in a provided opaque plastic box (the type used to store removable orthodontic devices). Volunteers were told to impregnate a sterile gauze with 5 mL of saline (the gauzes and the saline were provided as well) and extend it on the base of the plastic box, support the splints on it and close the box, leaving them at room temperature. The maximum allowed time that the volunteers had to eat and perform the oral hygiene measures were 20 minutes.

Using the permitted mechanical oral hygiene measures (without the splints), the volunteers performed the following mouthwash protocols based on the manufacturers' instructions, with the IDODS in the oral cavity, during the 4 days in the morning (8.30) after breakfast and at night (22.00) after dinner:

A) 20 mL mouthwashes for 30 seconds with essential oils containing alcohol/twice daily (4D-T-EO).

-OR-

B) 10 mL mouthwashes for 30 seconds with 0.2% chlorhexidine/twice daily (4D-0.2% CHX; positive control).

-OR-

C) 20 mL mouthwashes for 30 seconds with sterile water (4D-WATER; negative control).

A number was assigned to each participant (closed envelope) by an investigator who was unaware of the study design. Using an internet-based balanced randomisation system [33] introducing the numbers and the three test cycles (A, B and C), a random sequence indicating the mouthwash that each subject would use first, second and third was obtained. Although they were not told the type of mouthwash they were going to use, the obvious differences in taste between the three mouthwashes made allocation concealment to the volunteer impossible. The antiseptics/control were prepared in opaque bottles labelled with an A, B, or C depending on the containing solution, with 10 mL more than the quantity needed for completing the whole series of mouthwashes. The day before the start of

each experiment, after the full mouth dental scaling and polishing, participants were given by a masked investigator the corresponding previously made splints, the allocated opaque bottle, a plastic glass and a sterile 20 mL serum syringe with the objective of being the more precise possible with the quantity of solution used for the mouthwash. The day of the sample analysis, they were asked to bring back the bottles to measure the volume of solution left in the bottle. All volunteers performed the three rinsing cycles, with a rest period of 2 weeks between each test (Figure 2.2).

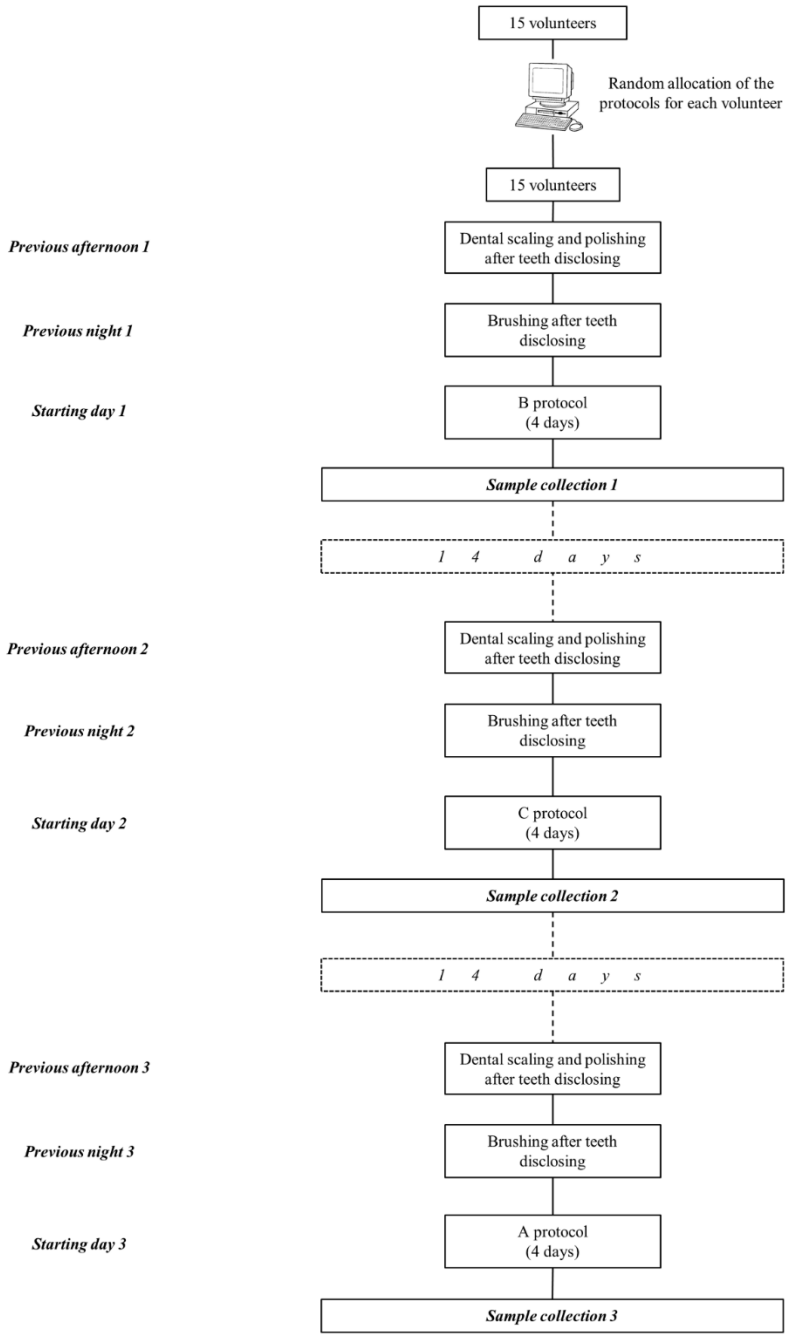

Figure 2.2. Protocol of the study.

2.3.4. PROCESSING OF THE SAMPLES OF PL-BIOFILM

Sample collection was done individually at the Unit of Confocal Microscopy of the Universidade de Santiago de Compostela at 8 AM in the morning so that the samples of each volunteer were analysed on different days. It was determined that a minimum of 10 hours should have elapsed since the last mouthwash on the previous night.

As the glass disks (in total, six) were removed from the splint, they were immediately immersed in 100 µL of fluorescence solution LIVE/DEAD® BacLight™ and kept in the dark chamber at room temperature for 15 minutes. The characteristics of LIVE/DEAD® BacLight™ fluorescence solution (Molecular Probes, Leiden, the Netherlands), as well as its preparation, have been described by the authors in a previous paper [34]. Microscopic observation was performed by a single investigator who was unaware of the study design, using a Leica TCS SP2 laser scanning spectral confocal microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) with an HCX APOL 63x/0.9 water-immersion lens.

Four selected fields or XYZ series in the central part of each disk were evaluated. These fields were considered representative of the whole sample after the observer's general examination. Fluorescence emission was determined in a series of XY images in which each image corresponded to each of the Z positions (depth). The optical sections were scanned in 1 µm sections from the surface of the biofilm to its base, measuring the maximum thickness of the field and subsequently the mean thickness of the biofilm of the corresponding sample. The maximum thickness of biofilm field was defined as the distance between the substrate (in perpendicular) and the peaks of the highest cell clusters [37]. The

maximum biofilm thickness of each field was divided into three zones or equivalent layers: the outer layer (layer 1), the middle layer (layer 2) and the inner layer (layer 3) (Figure 2.3).

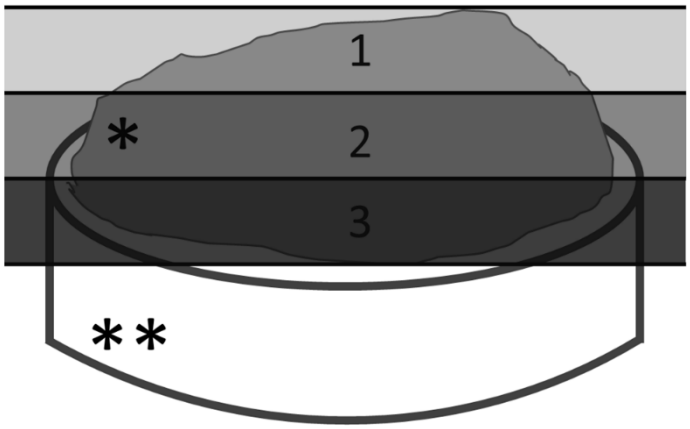

Figure 2.3. Scheme of the divisions of the PL-biofilm in layers. 1-Outer layer; 2-Middle layer; 3-Inner layer. *PL-biofilm. **Glass disk (substrate).

The capture of the data was done with the same settings in all cases, according to previously presented parameters in the precedent objective (Objective 1).

Quantification of BV was also done as previously presented using the cytofluorographic analysis (Leica Confocal Software) of XY images. In this analysis, the images of each fluorochrome were defined as “channels” (SYTO 9 occupies the green channel and PI the red channel). Square capture masks were used to measure the area occupied (μm^2) by the pixels in each channel, determining the total area occupied by the biofilm and the corresponding percentage of viability. The intensity range was considered a positive signal if it was between 100 and 255. Determination of the mean percentage of BV in each field required sections with a minimum area of biofilm of 250 μm^2; the mean percentage of BV of the biofilm was calculated for the corresponding sample and each biofilm layer.

For quantification of the percentage of the surface substrate covered by the biofilm (CG), the cytofluorogram itself was used. From the maximum projection (superposition of all planes captured) of each of the analysed fields, the percentage of CG was obtained by calculating the sum of the bacterial mass (viable and non-viable) regarding the total surface of the field (% positive within the total area).

2.3.5. STATISTICAL ANALYSIS

The results were analysed using the PASW® Statistics Base 20 package for Windows (IBM, Madrid, Spain) by an investigator who was blinded to the type of interventions analysed. All values from the quantitative variables examined (thickness, CG and BV) presented a normal distribution, which was determined using the Kolmogorov-Smirnov test. One-way ANOVA with repeated measures was used for intra-mouthwash (differentiating between the three biofilm layers) comparisons using all of the PL-biofilm samples. Two-way ANOVA with repeated measures was used for inter-mouthwash (distinguishing between the three biofilm layers) comparisons using all of the PL-biofilm samples. Pairwise comparisons (with the Bonferroni adjustment) were used for the analysis of intra- and inter-mouthwash results (including differentiating between the three biofilm layers). Statistical significance was taken as a *p* value less than 0.05.

2.4. RESULTS

A total of 34 volunteers were evaluated for eligibility to achieve the calculated sample size (n = 15). When this number of participants meeting the inclusion and exclusion criteria was reached, the enrollment process was stopped. A total of 19 subjects were not selected as eligible for not fulfilling the inclusion criteria. In relation to demographic characteristics of

the chosen participants, eight were females and seven males with a mean age of 25.4 ± 2.3 years. No adverse or side effects were observed by investigators or reported by the volunteers after the completion of any of the rising protocols.

2.4.1. INFLUENCE OF A 4-DAY PROTOCOL OF MOUTHWASHES OF TRADITIONAL ESSENTIAL OILS SOLUTION ON THE THICKNESS, COVERING GRADE AND BACTERIAL VIABILITY OF THE PL-BIOFILM

Both 4D-T-EO and 4D-0.2% CHX protocols were found to be significantly effective in regard to the 4D-WATER for reducing the biofilm thickness (9.99 ± 3.27 µm and 6.48 ± 1.82 µm respectively *vs.* 23.44 ± 4.78 µm; $p<0.001$, for all comparisons) (Figure 2.4). The CG was also significantly reduced by the 4D-T-EO and 4D-0.2% CHX in regard to the 4D-WATER (54.32 ± 17.49% and 20.01 ± 16.52%, respectively *vs.* 75.17 ± 16.51%; $p<0.05$ and $p<0.001$, respectively) (Figure 2.5).

When we compare both antiseptic solutions, the 0.2% CHX presented a higher activity than T-EO when reducing the PL-biofilm thickness ($p<0.05$) and the CG ($p<0.001$) after a period of 4 days (Figures 2.4 and 2.5).

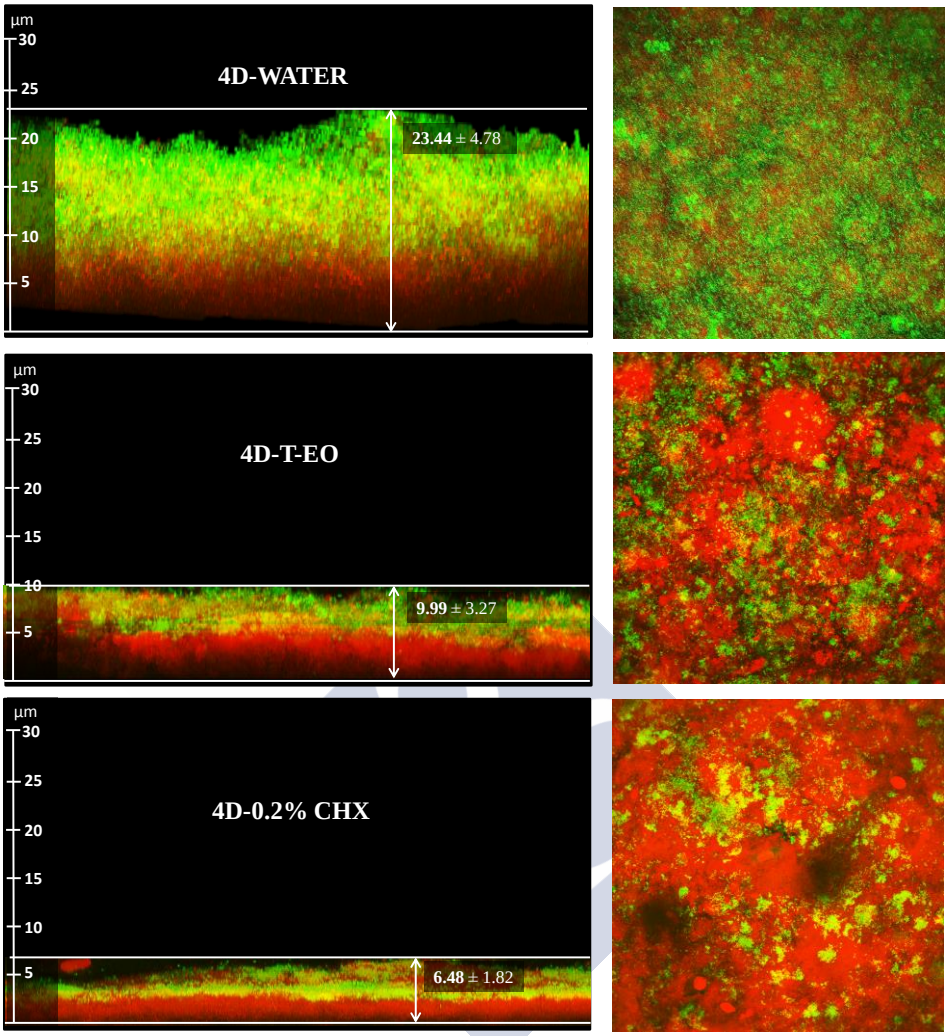

Figure 2.4. On the left, lateral projections of the brightest point (X-Z) from 4-day PL-biofilm images stacked in Y plane from the three rinsing protocols, presenting their respective mean thickness. On the right, maximum projections of the brightest point (X-Y) from 4-day PL-biofilm images stacked in Z plane. 4D-WATER = 4-day period during which mouthwashes with 20 mL of sterile water are done twice a day; 4D-T-EO = 4-day period during which mouthwashes with 20 mL of essential oils with alcohol are done twice a day; 4D-0.2% CHX = 4-day period during which mouthwashes with 10 mL of 0.2% chlorhexidine are done twice a day.

Both 4D-T-EO and 4D-0.2% CHX protocols were found to be significantly effective in regard to the 4D-WATER for reducing the BV (14.67 ± 5.54% and 13.19 ± 18.09%, respectively *vs.* 56.53 ± 14.40%; $p<0.001$)

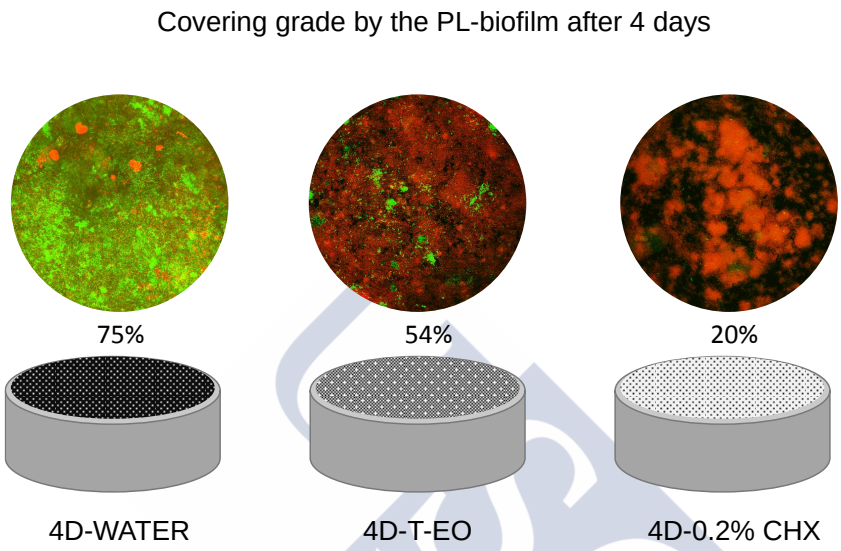

Figure 2.5. Representative images and graphics of the disks covering grade by the PL-biofilm after 4 days of continuous use of sterile water (4D-WATER), traditional essential oils (4D-T-EO) and 0.2% chlorhexidine (4D-0.2% CHX).

When we compare both antiseptic solutions, the EO was as effective as 0.2% CHX with regard to reducing the BV after 4 days of rinsing (Figure 2.6).

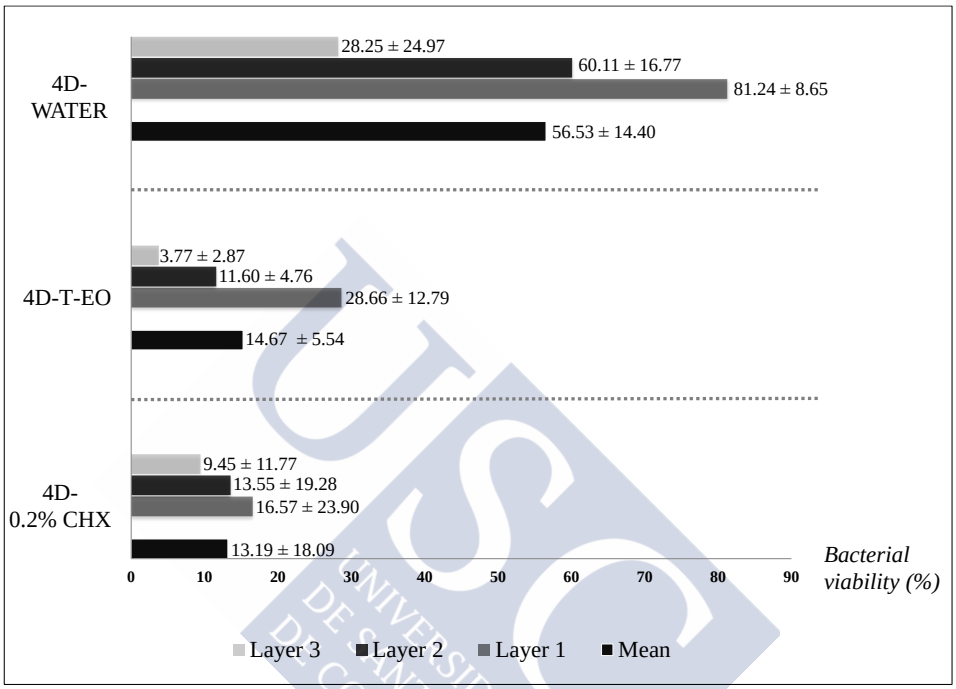

Figure 2.6. Bacterial viability in percentages of the 4-day PL-biofilm. Total and different layers in each rinsing protocol. 4D-WATER = 4-day period during which mouthwashes with 20 mL of sterile water are done twice a day; 4D-T-EO = 4-day period during which mouthwashes with 20 mL of essential oils with alcohol are done twice a day; 4D-0.2% CHX = 4-day period during which mouthwashes with 10 mL of 0.2% chlorhexidine are done twice a day. Layer 1 = the outer layer; Layer 2 = the middle layer; Layer 3= the inner layer.

In relation to the BV by layers, after the negative control and 4D-T-EO protocols, the BV was statistically higher (p<0.001) in the outer layers than in the inner ones. On the other hand, following the 4D-0.2% CHX period, there were no differences regarding BV among the three layers, and although it was higher in the outer layer, it did not reach statistical significance (Table 2.1).

Table 2.1. Results derived from mouthwash and inter-mouthwash comparisons on 4-day PL-biofilm bacterial viability divided into layers

PL-biofilm BACTERIAL VIABILITY DIVIDED INTO LAYERS			
INTRA-MOUTHWASH ANALYSIS			
	Layer 1 *vs.* Layer 2	Layer 1 *vs.* Layer 3	Layer 2 *vs.* Layer 3
4D-WATER	$p<0.001$	$p<0.001$	$p<0.001$
4D-T-EO	$p<0.001$	$p<0.001$	$p<0.001$
4D-0.2% CHX	---	---	---
INTER-MOUTHWASH ANALYSIS			
	Layer 1 *vs.* Layer 1	Layer 2 *vs.* Layer 2	Layer 3 *vs.* Layer 3
4D-WATER *vs.* 4D-T-EO	$p<0.001$	$p<0.001$	$p<0.05$
4D-WATER *vs.* 4D-0.2% CHX	$p<0.001$	$p<0.001$	$p<0.05$
4D-0.2% CHX *vs.* 4D-T-EO	---	---	---

---- Not a statistically significant difference.

4D-WATER = 4-day period during which mouthwashes with 20 mL of sterile water are done twice a day; 4D-T-EO = 4-day period during which mouthwashes with 20 mL of essential oils with alcohol are done twice a day; 4D-0.2% CHX = 4-day period during which mouthwashes with 10 mL of 0.2% chlorhexidine are done twice a day. Layer 1 = the outer layer; Layer 2 = the middle layer; Layer 3 = the inner layer.

In regard to the layer comparisons between the different rising protocols, both antiseptic solutions, T-EO and 0.2% CHX, presented less BV in all of them (Figure 2.6) in relation to the 4D-WATER period, with the differences being more marked between outer layers ($p<0.001$) and less pronounced ($p<0.05$) among the inner layers. There were no differences in terms of BV between the same layers after the 4D-T-EO and 4D-0.2% CHX regimes, being slightly higher in the outer layers in both cases.

2.5. DISCUSSION

2.5.1. METHODOLOGICAL APPROACH

The design of a 4-day model was chosen because it measures the growth of the PL-biofilm under the influence of a test solution, from a baseline of no dental plaque. If certain inhibition in the plaque formation cannot be demonstrated in this type of study, any significant effect must not be expected with a longer period of time [35].

There has been marked inter-individual variability detected regarding the characteristics of PL-biofilm [11, 23, 36]. In the present study, after the calculation of the sample size, a group of 15 individuals was selected. This sample group is in the line of similar studies in which the number of volunteers ranged from 7 to 24 [10, 23, 27, 28, 37].

In regard to the viability method selected, it has been stated that it was not possible to properly compare the viability assessed with fluorescence staining solutions with traditional plaque cultures. It is well known the important limitations of the latter method (among others, only 50% of the oral bacteria are culturable [38]), which emphasises the necessity of using viability assays [29]. The LIVE/DEAD® BacLight™ fluorescence assay stains the bacteria in red or green depending on the permeability of their membrane. Given that the tested antiseptics act mostly at this cellular element, this vital staining method is suitable for this type of study.

Although there has been some discussion about the reliability of this technique [29, 39, 40], reasons for its use when assessing BV in natural dental biofilms has been previously given (Objective 1). Furthermore, the fact of doing 24 different measures (four measures per disk; in total, six

disks) in every volunteer in each of the rising tests, being at the same time, a crossover study, considerably reduce the potential bias that could exist by the determination of the BV by this technique.

Given that all microbiological techniques have their disadvantages, and although the presented results are coherent with the clinical reality, the author recognises the convenience of contrasting and complementing the data obtained with BacLight™ fluorescence solution with other methods. These methods include the application of other fluorescent dyes, other molecular or bacteriological techniques or even with macroscopic clinical data, provided they have been achieved in a more objective way that the registration of conventional plaque indices.

2.5.2. INFLUENCE OF A 4-DAY PROTOCOL OF MOUTHWASHES OF TRADITIONAL ESSENTIAL OILS SOLUTION ON THE THICKNESS, COVERING GRADE AND BACTERIAL VIABILITY OF THE PL-BIOFILM

Many short-term studies have been published which assess the effect of T-EO and CHX (among other antiseptics) on oral biofilm *in situ* [14, 16, 17, 41]. The main disadvantage of this type of study is the fact that the dental plaque must be non-disturbed if some parameters like BV are to be measured, making it impossible to evaluate the architecture or actual thickness of the biofilm itself. This distortion would interfere in the delicate 3-D relationship existing between the bacteria inside the biofilm [11, 12].

In contrast, there are fewer short-term studies on undisturbed PL-biofilm evaluating antimicrobial agents like the CHX (analysed by TEM [37] or by CLSM [10, 23, 27, 42], amine/stannous fluoride [10] or zinc chloride [28]. On the contrary, to the best of author's knowledge, there are no published papers which compare the antiplaque effect of T-EO *vs.* 0.2%

CHX in an *in situ* model of 4-day PL-biofilm, analysing at the same time thickness, CG and BV on biofilm using CLSM techniques combined with fluorescence staining procedures.

- *Thickness and covering grade reduction*

In the present study, the obtained thickness was 23.44 µm after 4-day-growing PL-biofilm without any disturbing antiplaque agent. These results agree with those obtained by Jentsch et al., of 19.24 µm after 3 days [37], and Arweiler et al., of 25.33 µm after 5 days [27]. In contrast, they differ from those obtained by Gu et al., of 37 µm [28], and, to a greater extent, those from Auschill et al., of 76.7 µm [10], the latter two were after 2 days.

At this point, two issues should be commented upon; the first referring to the study of Jentsch et al. [37], in which they evaluated a 3 day-evolution of the PL-biofilm measured by TEM. Despite being very accurate, this technique requires the fixation and drying of the biofilm, with unavoidable consequences to its delicate structure. The second is a critical methodological aspect which should be taken into account when using CLSM for measuring PL-biofilm thicknesses and refers to the high thickness data obtained by Gu et al. [28] and mainly by Auschill et al. [10]. The method they used for thickness measurements is different from that used by other authors [27, 43] and the current study. They determined the PL-biofilm thickness using the number of 1-µm-planes obtained by CLSM (regardless of the perpendicularity); using a measurement like this could result in errors in many cases because a minimum substrate inclination could carry on an evaluation of the substrate in the diagonal, which would overestimate the thickness of the PL-biofilm obtained. In this paper, the Leica Confocal SPII software was used to get the exact distance existing between the substrate

and the highest point of the PL-biofilm perpendicularly, which is a more realistic measure.

The thickness of the *in situ* PL-biofilm after the 4-day application of 0.2% CHX and T-EO was 6.48 µm and 9.99 µm, respectively. There are several studies [10, 27, 28, 37, 44] in which the PL-biofilm thickness was analysed. The results differ between them depending on the technique used for its measurement (SEM, TEM or CLSM), type and concentration of the mouthwashes and its duration.

The only study apart from the present series that compares the efficacy of T-EO and CHX is the one from Jentsch et al. [44]. These authors measured the thickness of the PL-biofilm developed on the surface of enamel slabs after a rising period of 4 days. Unlike the findings reported in the present paper, they did not obtain any differences in the effect of reducing the PL-biofilm thickness between both antiseptic solutions, T-EO and CHX (15.13 *vs.* 16.67 after 4 days). However, it should be noted that the concentration of CHX used in our study was higher (0.2%) than that applied by Jentsch et al. (0.12%) [44]. Our results are similar to those obtained by other authors after the application of 0.2% CHX (8.6 µm after 2 days [10] and 11.91 µm after 5 days) [27]. When a lower concentration was applied (0.12% CHX) the thickness rose to 14.02 µm after 3 days (TEM) [37] and 16.67 µm after 4 days (SEM) [44]. After the using of amine/stannous fluoride, the thicknesses obtained were 15.7 µm after 2 days [10], 11.91 µm after 3 days (TEM) and 13.25 µm after 4 days (SEM) [37]. Finally, after 2-day use of zinc chloride at a concentration of 10 mM yielded a thickness of 10 µm [28].

The CG by the PL-biofilm in combination with its thickness is directly related to the antiplaque capacity of an antiseptic agent. This parameter is important because it can be predictive of the adaptation of microorganisms to environmental influences [45]. To the best of author's knowledge, there are no published papers on this issue in 4-day PL-biofilm *in situ*. On the other hand, there are some *in vitro* studies on this topic and one of them obtained similar results to the present series after 4 days. It is one from Al-Ahmad et al. [46], who obtained CG percentages of 77% and 7% with negative control and 0.2% CHX after 4-day use, respectively (in the present series, 75% and 20%, respectively).

In the present study, 0.2% CHX was more potent for inhibiting the PL-biofilm formation, since both the thickness and CG were considerably lower than those observed with the T-EO protocol.

- *Antibacterial activity*

In regard to the PL-biofilm viability, it was around the 56% after 4 days of growth in the present series. This contrasts with data reported in other similar studies where the viability was between 60% and 70% [10, 28], but this was described in a 2-day biofilm; these values are similar to those which the authors of this paper have obtained in previous studies on PL-biofilm after 48 hours [23, 47] (Objective 1). On the contrary, Arweiler et al. [27] obtained the same results (56.8%) in a PL-biofilm which was, presumably, more similar to ours because of its 5-day evolution. This lower BV is highly influenced by the deepest layer of the PL-biofilm (the nearest to the substrate), which is clearly where the differences are found in regard with the PL-biofilm with less time of evolution. Arweiler et al. [27] also detected a lower viability in the deepest layer, coinciding with the theory that the

bacteria located in the deepest part of a biofilm are in an inactive metabolic state [22, 48].

Concerning the BV after the mouthwash protocols with T-EO and 0.2% CHX, the BV reductions are similar to those which have been previously reported [10, 27].

In the literature, for 0.2% CHX and amine/stannous fluoride, these BV values were 62% and 64%, respectively [10, 27] and 43% for zinc chloride at a concentration of 10 mM [28]. In the present series, the BV reduction was similar between both antiseptics (74% for T-EO and 77% for 0.2% CHX). Once again, the lowest BV was found in the deepest layers of the PL-biofilm.

In the present series, the results obtained both in thickness and BV for 0.2% CHX were considerably lower than those achieved by Arweiler et al. [27]. This result could be because of some noticeable methodological differences that should be discussed. In the present study, the volunteers did the negative and the test cycles in different periods, which permitted active mouthwashes being used *in situ* with the antiplaque agents. On the other hand, Arweiler et al. replaced the mouthwash with a simple immersion in the test solution (0.2% CHX), one hemiarch was immersed in the test solution and the other in the control. By doing this, they were assuming that an immersion was similar to an active mouthwash, ignoring the intrinsic factors that a rinse has itself such as the greatest washing effect due to the muscular force applied by the cheeks. In a previous paper, Auschill et al. [10] highlighted the importance of doing the mouthwash *in situ* because Pratten et al. [49] had previously exposed an *in vitro* biofilm to 0.2% CHX solution for 5 minutes, observing minimal effects on the biofilm. Based on

this, in a previous study, our research group noted that the effects of both 0.2% CHX and EO on the 2-day PL-biofilm were not comparable at all in the case of a passive immersion and an active mouthwash [50].

There are two 4-day *in situ* studies which compare the effect of T-EO and CHX in terms of bacterial counts [15, 51]. In these studies, the plaque control was done, and culturable species were determined using plaque culture techniques. Eventually, they concluded that T-EO and 0.12% or 0.1 % CHX (the concentrations used, respectively) had similar antiplaque effects. In the present series, although T-EO and 0.2% CHX showed a very high and similar antibacterial activity, the latter was more powerful when inhibiting the PL-biofilm formation, since both the thickness and CG were considerably lower in its case.

2.5.3. CLINICAL STUDIES ON THE ANTIPLAQUE EFFECT OF THE TRADITIONAL ESSENTIAL OILS SOLUTION

Results obtained in microbiological studies, including the present paper, should be contrasted with those derived from clinical studies, based on the application of antiseptics in the short and long term. The findings found in the present series are consistent with those from Riep et al. [13] and Pizzo et al. [41]. These two studies are clinical trials on the antiplaque activity of different antiseptics after 4 days, where both T- EO and CHX were effective in reducing the amount of dental plaque on the tooth surface regarding the negative control. In this sense, it should be mentioned that in several literature reviews it has been concluded that performing daily mouthwashes with T-EO produces a similar antiplaque effect to that of CHX [7, 52, 53]. On the contrary, in a relatively recent revision, Neely et al. [54] demonstrated that although the effect of the T-EO and the 0.2% CHX at

reducing gingivitis may be equivalent, the latter is more effective in decreasing plaque formation after 6 months of use.

Although both the T-EO and the CHX has proved antiseptic efficacy on the *in situ* PL-biofilm, when are used for long periods, some clinicians have to deal with possible side effects of both. In the case of CHX, they are mainly dental staining [5] and taste alterations. When prescribing T-EO for long periods, there is always the controversy of using alcohol containing mouthwashes due to its possible (remains still unproven [55]) relation with the risk of developing oral cancer. For these reasons, a possible alternative could be the commercial formulas of EO without alcohol, so it would be interesting a new objective based on testing the EO without alcohol with this same model *in situ*, to evaluate its antibacterial activity and antiplaque efficacy in the short term before testing it in the long term.

2.6. CONCLUSION

In a 4-day *in situ* undisturbed plaque-like biofilm model, the traditional formula based on essential oils showed a very high antiplaque effect. Essential oils had a very high antibacterial activity and similar to that detected with the 0.2% chlorhexidine, although the latter presented better results at reducing the thickness and covering grade by the plaque-like biofilm.

Consequently, the traditional essential oils solution is a reliable alternative to chlorhexidine to prevent its side effects when used continuously.

2.7. REFERENCES

1. **Loe, H., Theilade, E., and Jensen, S.B.**, *Experimental Gingivitis in Man.* J Periodontol, 1965. 36: p. 177-87.

2. **Tatakis, D.N. and Trombelli, L.**, *Modulation of clinical expression of plaque-induced gingivitis. I. Background review and rationale.* J Clin Periodontol, 2004. 31(4): p. 229-38.

3. **Rodrigues, J.A., dos Santos, P.A., Garcia, P.P., Corona, S.A., and Loffredo, L.C.**, *Evaluation of motivation methods used to obtain appropriate oral hygiene levels in schoolchildren.* Int J Dent Hyg, 2003. 1(4): p. 227-32.

4. **Demke, R.**, *Plaque inhibition: the science and application of oral rinses.* Dent Today, 2012. 31(2): p. 96-101; quiz 101, 95.

5. **Van Strydonck, D.A., Slot, D.E., Van der Velden, U., and Van der Weijden, F.**, *Effect of a chlorhexidine mouthrinse on plaque, gingival inflammation and staining in gingivitis patients: a systematic review.* J Clin Periodontol, 2012. 39(11): p. 1042-55.

6. **Wu, C.D. and Savitt, E.D.**, *Evaluation of the safety and efficacy of over-the-counter oral hygiene products for the reduction and control of plaque and gingivitis.* Periodontol 2000, 2002. 28: p. 91-105.

7. **Charles, C.H., Mostler, K.M., Bartels, L.L., and Mankodi, S.M.**, *Comparative antiplaque and antigingivitis effectiveness of a chlorhexidine and an essential oil mouthrinse: 6-month clinical trial.* J Clin Periodontol, 2004. 31(10): p. 878-84.

8. **Sharma, N., Charles, C.H., Lynch, M.C., Qaqish, J., McGuire, J.A., Galustians, J.G., and Kumar, L.D.**, *Adjunctive benefit of an essential oil-containing mouthrinse in reducing plaque and gingivitis in patients who brush and floss regularly: a six-month study.* J Am Dent Assoc, 2004. 135(4): p. 496-504.

9. **Fine, D.H., Furgang, D., and Barnett, M.L.**, *Comparative antimicrobial activities of antiseptic mouthrinses against isogenic planktonic and biofilm forms of Actinobacillus actinomycetemcomitans.* J Clin Periodontol, 2001. 28(7): p. 697-700.

10. **Auschill, T.M., Hein, N., Hellwig, E., Follo, M., Sculean, A., and Arweiler, N.B.**, *Effect of two antimicrobial agents on early in situ biofilm formation.* J Clin Periodontol, 2005. 32(2): p. 147-52.

11. **Wood, S.R., Kirkham, J., Marsh, P.D., Shore, R.C., Nattress, B., and Robinson, C.**, *Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy.* J Dent Res, 2000. 79(1): p. 21-7.

12. **Beyth, N., Yudovin-Farber, I., Perez-Davidi, M., Domb, A.J., and Weiss, E.I.**, *Polyethyleneimine nanoparticles incorporated into resin composite cause cell death and trigger biofilm stress in vivo.* Proc Natl Acad Sci U S A, 2010. 107(51): p. 22038-43.

13. **Riep, B.G., Bernimoulin, J.P., and Barnett, M.L.**, *Comparative antiplaque effectiveness of an essential oil and an amine fluoride/stannous fluoride mouthrinse.* J Clin Periodontol, 1999. 26(3): p. 164-8.

14. **Arweiler, N.B., Boehnke, N., Sculean, A., Hellwig, E., and Auschill, T.M.**, *Differences in efficacy of two commercial 0.2% chlorhexidine mouthrinse solutions: a 4-day plaque re-growth study.* J Clin Periodontol, 2006. 33(5): p. 334-9.

15. **Rosin, M., Welk, A., Kocher, T., Majic-Todt, A., Kramer, A., and Pitten, F.A.**, *The effect of a polyhexamethylene biguanide mouthrinse compared to an essential oil rinse and a chlorhexidine rinse on bacterial counts and 4-day plaque regrowth.* J Clin Periodontol, 2002. 29(5): p. 392-9.

16. **Pizzo, G., La Cara, M., Licata, M.E., Pizzo, I., and D'Angelo, M.**, *The effects of an essential oil and an amine fluoride/stannous fluoride mouthrinse on supragingival plaque regrowth.* J Periodontol, 2008. 79(7): p. 1177-83.

17. **Singh, A., Daing, A., and Dixit, J.**, *The effect of herbal, essential oil and chlorhexidine mouthrinse on de novo plaque formation.* Int J Dent Hyg, 2013. 11(1): p. 48-52.

18. **Dige, I., Nilsson, H., Kilian, M., and Nyvad, B.**, *In situ identification of streptococci and other bacteria in initial dental biofilm by confocal laser scanning microscopy and fluorescence in situ hybridization.* Eur J Oral Sci, 2007. 115(6): p. 459-67.

19. **Costerton, J.W., Lewandowski, Z., DeBeer, D., Caldwell, D., Korber, D., and James, G.**, *Biofilms, the customized microniche.* J Bacteriol, 1994. 176(8): p. 2137-42.

20. **Donlan, R.M. and Costerton, J.W.**, *Biofilms: survival mechanisms of clinically relevant microorganisms.* Clin Microbiol Rev, 2002. 15(2): p. 167-93.

21. **Hahnel, S., Rosentritt, M., Burgers, R., and Handel, G.**, *Surface properties and in vitro Streptococcus mutans adhesion to dental resin polymers.* J Mater Sci Mater Med, 2008. 19(7): p. 2619-27.

22. **Netuschil, L., Reich, E., Unteregger, G., Sculean, A., and Brecx, M.**, *A pilot study of confocal laser scanning microscopy for the assessment of undisturbed dental plaque vitality and topography.* Arch Oral Biol, 1998. 43(4): p. 277-85.

23. **García-Caballero, L., Quintas, V., Prada-López, I., Seoane, J., Donos, N., and Tomás, I.**, *Chlorhexidine substantivity on salivary flora and plaque-like biofilm: an in situ model.* PLoS One, 2013. 8(12): p. e83522.

24. **Auschill, T.M., Arweiler, N.B., Netuschil, L., Brecx, M., Reich, E., and Sculean, A.**, *Spatial distribution of vital and dead microorganisms in dental biofilms.* Arch Oral Biol, 2001. 46(5): p. 471-6.

25. **Hope, C.K. and Wilson, M.**, *Biofilm structure and cell vitality in a laboratory model of subgingival plaque.* J Microbiol Methods, 2006. 66(3): p. 390-8.

26. **Korber, D.R., Lawrence, J.R., Hendry, M.J., and Caldwell, D.E.**, *Analysis of spatial variability within mot+ and mot- Pseudomonas fluorescens biofilms using representative elements.* Biofouling, 1993. 7: p. 339-358.

27. **Arweiler, N.B., Lenz, R., Sculean, A., Al-Ahmad, A., Hellwig, E., and Auschill, T.M.**, *Effect of food preservatives on in situ biofilm formation.* Clin Oral Investig, 2008. 12(3): p. 203-8.

28. **Gu, H., Fan, D., Gao, J., Zou, W., Peng, Z., Zhao, Z., Ling, J., and LeGeros, R.Z.**, *Effect of ZnCl2 on plaque growth and biofilm vitality.* Arch Oral Biol, 2012. 57(4): p. 369-75.

29. **Tawakoli, P.N., Al-Ahmad, A., Hoth-Hannig, W., Hannig, M., and Hannig, C.**, *Comparison of different live/dead stainings for detection and quantification of adherent microorganisms in the initial oral biofilm.* Clin Oral Investig, 2013. 17(3): p. 841-50.

30. **Pan, P., Barnett, M.L., Coelho, J., Brogdon, C., and Finnegan, M.B.**, *Determination of the in situ bactericidal activity of an essential oil mouthrinse using a vital stain method.* J Clin Periodontol, 2000. 27(4): p. 256-61.

31. **Faul, F., Erdfelder, E., Lang, A.G., and Buchner, A.**, *G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences.* Behav Res Methods, 2007. 39(2): p. 175-91.

32. **World Health Organization**, *Oral health surveys: basic methods*. 5th ed. 2013, France. 125.

33. **Dallal, G.E.**, Available from: www.randomization.com. Last access on 03/09/2017.

34. **Tomás, I., García-Caballero, L., Cousido, M.C., Limeres, J., Álvarez, M., and Diz Dios, P.**, *Evaluation of chlorhexidine substantivity on salivary flora by epifluorescence microscopy.* Oral Dis, 2009. 15(6): p. 428-33.

35. **Moran, J., Addy, M., Kohut, B., Hovliaras, C.A., and Newcombe, R.G.**, *Efficacy of mouthrinses in inhibiting the development of supragingival plaque over a 4-day period of no oral hygiene.* J Periodontol, 1994. 65(10): p. 904-7.

36. **Arweiler, N.B., Hellwig, E., Sculean, A., Hein, N., and Auschill, T.M.**, *Individual vitality pattern of in situ dental biofilms at different locations in the oral cavity.* Caries Res, 2004. 38(5): p. 442-7.

37. **Jentsch, H., Hombach, A., Beetke, E., and Jonas, L.**, *Quantitative transmission electron microscopic study of dental plaque--an in vivo study with different mouthrinses.* Ultrastruct Pathol, 2002. 26(5): p. 309-13.

38. **Aas, J.A., Paster, B.J., Stokes, L.N., Olsen, I., and Dewhirst, F.E.**, *Defining the normal bacterial flora of the oral cavity.* J Clin Microbiol, 2005. 43(11): p. 5721-32.

39. **Hannig, C., Follo, M., Hellwig, E., and Al-Ahmad, A.**, *Visualization of adherent micro-organisms using different techniques.* J Med Microbiol, 2010. 59(Pt 1): p. 1-7.

40. **Netuschil, L., Auschill, T.M., Sculean, A., and Arweiler, N.B.**, *Confusion over live/dead stainings for the detection of vital microorganisms in oral biofilms - which stain is suitable?* BMC Oral Health, 2014. 14: p. 2.

41. **Pizzo, G., Compilato, D., Di Liberto, B., Pizzo, I., and Campisi, G.**, *Effects of two essential oil mouthrinses on 4-day supragingival plaque regrowth: a randomized cross-over study.* Am J Dent, 2013. 26(3): p. 156-60.

42. **Tomás, I., García-Caballero, L., López-Álvar, E., Suárez, M., Diz, P., and Seoane, J.**, *In situ chlorhexidine substantivity on saliva and plaque-like biofilm: influence of circadian rhythm.* J Periodontol, 2013. 84(11): p. 1662-72.

43. **Roberts, S.K., Bass, C., Brading, M., Lappin-Scott, H., and Stoodley, P.**, *Biofilm formation and structure; What's new?*, in *Dental plaque revisited-oral biofilms in health and disease*, W.M. Newman HN, Editor. 1999, BioLine: Cardiff. p. 1-36.

44. **Jentsch, H., Mozaffari, E., and Jonas, L.**, *Scanning electron microscopy of growing dental plaque: a quantitative study with different mouth rinses.* Ultrastruct Pathol, 2013. 37(4): p. 233-40.

45. **Al-Ahmad, A., Wiedmann-Al-Ahmad, M., Carvalho, C., Lang, M., Follo, M., Braun, G., Wittmer, A., Mulhaupt, R., and Hellwig, E.**, *Bacterial and Candida albicans adhesion on rapid prototyping-produced 3D-scaffolds manufactured as bone replacement materials.* Journal Of Biomedical Materials Research Part A, 2008. 87(4): p. 933-43.

46. **Al-Ahmad, A., Wiedmann-Al-Ahmad, M., Auschill, T.M., Follo, M., Braun, G., Hellwig, E., and Arweiler, N.B.**, *Effects of commonly used food preservatives on biofilm formation of Streptococcus mutans in vitro.* Arch Oral Biol, 2008. 53(8): p. 765-72.

47. **Prada-López, I., Quintas, V., Donos, N., Suárez-Quintanilla, D., and Tomás, I.**, *Characteristics of in situ oral biofilm after 2 and 4 days of evolution.* Quintessence Int, 2015. 46(4): p. 287-298.

48. **Koo, H., Nino de Guzman, P., Schobel, B.D., Vacca Smith, A.V., and Bowen, W.H.**, *Influence of cranberry juice on glucan-mediated processes involved in Streptococcus mutans biofilm development.* Caries Res, 2006. 40(1): p. 20-7.

49. **Pratten, J., Barnett, P., and Wilson, M.**, *Composition and susceptibility to chlorhexidine of multispecies biofilms of oral bacteria.* Appl Environ Microbiol, 1998. 64(9): p. 3515-9.

50. **Prada-López, I., Quintas, V., Casares-De-Cal, M.A., Suárez-Quintanilla, J.A., Suárez-Quintanilla, D., and Tomás, I.**, *Ex vivo vs. in vivo antibacterial activity of two antiseptics on oral biofilm.* Front Microbiol, 2015. 6: p. 655.

51. **Ulkur, F., Arun, T., and Ozdemir, F.**, *The effects of three different mouth rinses in a 4-day supragingival plaque regrowth study.* Eur J Dent, 2013. 7(3): p. 352-8.

52. **Stoeken, J.E., Paraskevas, S., and van der Weijden, G.A.**, *The long-term effect of a mouthrinse containing essential oils on dental plaque and gingivitis: a systematic review.* J Periodontol, 2007. 78(7): p. 1218-28.

53. **Van Leeuwen, M.P., Slot, D.E., and Van der Weijden, G.A.**, *Essential oils compared to chlorhexidine with respect to plaque and parameters of gingival inflammation: a systematic review.* J Periodontol, 2011. 82(2): p. 174-94.

54. **Neely, A.L.**, *Essential oil mouthwash (EOMW) may be equivalent to chlorhexidine (CHX) for long-term control of gingival inflammation but CHX appears to perform better than EOMW in plaque control.* J Evid Based Dent Pract, 2012. 12(3 Suppl): p. 69-72.

55. **Gandini, S., Negri, E., Boffetta, P., La Vecchia, C., and Boyle, P.**, *Mouthwash and oral cancer risk quantitative meta-analysis of epidemiologic studies.* Ann Agric Environ Med, 2012. 19(2): p. 173-80.

OBJECTIVE 3

Quintas, V.; Prada-López, I.; Carreira, MJ.; Suárez-Quintanilla, D.; Balsa-Castro, C.; Tomás, I. *In situ* antibacterial activity of essential oils with and without alcohol on oral biofilm: a randomized clinical trial. *Front Microbiol*. 2017. 8:2162.

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OBJECTIVE 3. ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS WITH AND WITHOUT ALCOHOL ON AN *IN SITU* MODEL OF UNDISTURBED ORAL BIOFILM: A RANDOMISED CLINICAL TRIAL

3.1. ABSTRACT

Introduction: Currently, there is little evidence on the *in situ* antibacterial activity of essential oils (EO) without alcohol.

Objective: This study aimed to evaluate *in situ* the substantivity and antiplaque effect on the plaque-like biofilm (PL-biofilm) of two solutions: a traditional formulation that contains EO with alcohol (T-EO) and an alcohol-free formulation of EO (Af-EO).

Material and Methods: Eighteen healthy adults performed a single mouthwash of T-EO, Af-EO, and sterile water (WATER) after wearing an individualised disk-holding splint for 2 days. The thickness and the bacterial viability (BV) of the PL-biofilm were quantified at baseline, 30 seconds, and 1, 3, 5 and 7 hours post-rinsing (Test 1). Subsequently, each volunteer wore the splint for 4 days, applying two daily mouthwashes of T-EO, Af-EO and WATER. The thickness, covering grade (CG) and BV, of the PL-biofilm were quantified (Test 2). Samples were analysed by confocal laser scanning microscopy (CLSM) after staining with the LIVE/DEAD® BacLight™ solution. In order to conduct the computations of the BV automatically, a Matlab toolbox called Dentius biofilm was developed. This Clinical Trial was registered at clinicaltrials.gov with the number NCT03146390 URL: https://clinicaltrials.gov/ct2/show/NCT03146390.

Results: The thickness of the PL-biofilm after rinsing was not affected by any of the formulations and ranged from 18 to 24 µm. The T-EO and Af-EO solutions had a similar antibacterial effect, reducing BV after a single mouthwash compared to the WATER, and keeping it below baseline levels up to 7 hours post-rinse (p<0.001) After 4 days, both antiseptics were significantly more effective than the WATER, reducing the thickness, CG and BV of the PL-biofilm. The T-EO rinse presented slightly higher bactericidal activity than the Af-EO solution, although no significant results were achieved (BV = 26.27% *vs.* 31.08%). The Af-EO rinse, however, led to more significant reductions in the thickness (9.92 µm *vs.* 7.90 µm, p = 0.012) and CG of the PL-biofilm (46.61% *vs.* 33.36%, p<0.016).

Conclusion: Both EO antiseptics had very high immediate antibacterial activity and substantivity *in situ* on the 2-day PL-biofilm after a single mouthwash. In the 4-day PL-biofilm, both essential oil formulations demonstrated a very good antiplaque effect *in situ*, although the alcohol-free formula performed better at reducing the biofilm thickness and covering grade.

Clinical Relevance: The alcohol-free essential oil solution represents a reliable option as antibacterial and antiplaque agent for the control of oral biofilm.

3.2. INTRODUCTION

The accumulation of bacterial biofilms on tooth surfaces results in two of the most prevalent infectious diseases – caries and periodontitis. Although prevention and control of these diseases can be achieved by the daily mechanical removal of biofilms, many people are either unable or unwilling to practice these procedures as regularly or as efficiently as necessary. There is, therefore, considerable interest in the possibility of using chemicals to replace or augment, mechanical preventive and therapeutic procedures [1-3].

The active ingredients present in the mouthwashes that are most commonly employed in the oral cavity include: chlorhexidine (CHX), combinations of essential oils (EO), triclosan, cetylpyridinium chloride, and various metal salts such as zinc compounds and stannous fluoride. Of all of these, CHX mouthwashes are considered to be the *Gold Standard*, as they have thus far been the most effective in microbiological and clinical studies [4-6]. However, their well-known undesirable secondary effects, mainly after regular use [7], have led to the scientific community exploring the existence of effective alternatives, especially when daily use is required. Accordingly, EO have been found to be as effective as CHX at controlling gingival inflammation after 6 months of use, although the latter performs better at reducing plaque levels [8, 9].

The EO are composed of a wide variety of products. As a consequence, their antimicrobial activity is related to their composition, configuration, amount and possible interactions [10]. The traditional formulations containing EO (T-EO) (Listerine® Mentol™, Johnson & Johnson) are a complex mix of phenolic compounds combined with various EO: 0.092%

eucalyptol, 0.064% thymol, 0.06% methyl-salicylate, and 0.042% menthol. All of these are included in a hydroalcoholic vehicle containing between 21.6% and 26.9% alcohol [11]. As a result, EO contains ethanol, which is a chemical compound used to dissolve and stabilise the numerous substances present in the rinse. The concentration of ethanol present in the EO rinses is more than 20%. Such amounts have been found to be sufficient to dissolve the EO, but insufficient when it comes to having a direct antibacterial effect [12, 13]. In fact, the manufacturer presents the alcohol contained in the rinse (21.6%) as being, inter alia, an inactive ingredient in its formula [14]. Over the years, the use of ethanol in mouthwashes, as well as their effects on the surfaces of composite restorations [15] and their possible role in the development of oropharyngeal cancer, have been discussed [16, 17]. A direct cause-and-effect association between the development of oropharyngeal carcinoma and the use of alcohol-containing rinses has not been demonstrated [18-20], and probably never will be (at least by epidemiological studies) [21]. However, it is considered desirable to eliminate ethanol from daily mouthwashes, especially for paediatric populations and patients at higher risk for oral cancer [22, 23]. Furthermore, the fact that the alcohol is present has meant that some clinical practitioners do not prescribe the T-EO due to this controversy [24]. All of this has led to the development of new, alcohol-free formulations of EO (Af-EO) (Listerine® Zero™, Johnson & Johnson).

The composition of Af-EO is the same in terms of their active ingredients (eucalyptol, thymol, methyl-salicylate and menthol), but sodium fluoride has been added to the mixture. Some differences are found in their inactive ingredients. These are based on the alcohol contained in the T-EO solutions, which is not present in the Af-EO rinses, and the presence of

propylene glycol, sodium lauryl sulphate and sucralose in the Af-EO solutions, but not the T-EO rinses.

Two different concepts should be taken into account to measure the efficacy of a mouthwash against dental plaque: the substantivity and the antiplaque effect. The substantivity of an oral antiseptic is defined as the prolonged adherence to oral surfaces (teeth and mucosa) and its slow release at active doses which guarantee the continuation of the antimicrobial activity [25]. The more substantivity an oral antiseptic has, the better. To study this *in situ*, the most popular models are those that analyse the effect that a single mouthwash has on a biofilm of more than 24 hours [26].

The second concept that should be studied, the antiplaque effect, is defined as the capacity that an agent has to prevent the formation of bacterial aggregates (plaque) on oral surfaces. To study this *in situ*, models start from a baseline sample with levels of plaque near to zero to assess the power of the antiseptic when it comes to reducing the formation of bacterial plaque (usually dental plaque) compared to the control. A 6-month clinical study using a determinate antiplaque agent is necessary to tag an antiseptic as effective [27]. However, in the literature, there is an established model of 4 days of plaque regrowth with which authors can assess the inhibitory activity that different mouthwashes have*;* furthermore, this determines the relative efficacy of the different formulations as being predictive of the antiplaque effect of an antiseptic [28].

Also, another important methodological aspect in the *in situ* research of an oral antiseptic is the need to conserve the oral biofilm intact at all stages, namely the formation, collection and analysis of the oral samples. The goal is to not interfere with the delicate three- dimensional (3-D)

structure of the oral biofilm, which has been proved to be essential regarding the resistance to the effects of an antimicrobial agent [29, 30]. For these reasons, the use of intraoral disks held in specially designed apparatus for biofilm formation combined with the application of confocal laser scanning microscopy (CLSM) has proved to be extremely valuable when it comes to analysing the oral biofilm in its intact, hydrated natural state [26, 31-33].

As Af-EO have come to the market, it seems appropriate to compare their antibacterial effects to those of traditional mouthwashes. Although there are some studies evaluating these effects of T-EO and Af-EO [13, 34-39], none of them has assessed and compared their substantivity and antiplaque impact in an *in situ* model of undisturbed plaque-like biofilm (PL-biofilm). For this reason, the aim of the present study was to compare the *in situ* antibacterial activity (immediate effect, substantivity and antiplaque effect) of EO with and without alcohol on the PL-biofilm.

3.3. MATERIAL AND METHODS

This investigation is a randomised, double blind, crossover study of the antibacterial and antiplaque efficacy of two available formulas based on EO: traditional EO with alcohol in the formulation (T-EO) and alcohol-free EO (Af-EO). The study meets the CONSORT checklist points and received the approval of the Clinical Research Ethics Committee of Galicia (number 2014/008. It was registered at clinicaltrials.gov with the number NCT03146390. URL: https://clinicaltrials.gov/ct2/show/NCT03146390.

The ‘a priori’ sample size calculation was performed using the program G*Power 3.1.5 [40]. The following statistical criteria were established: 1) an effect size of 0.7; 2) an alpha error of 0.05; and 3) a statistical power of 80%.

A sample size of 19 subjects was required by these criteria and the application of the Wilcoxon test to analyse the differences in the microscopic parameters between two rinsing protocols.

The participants were selected among dental students at the Faculty of Medicine and Dentistry of Santiago de Compostela (Universidade de Santiago de Compostela, Spain), where volunteer enrollment was sought by inviting responses to advertisements displayed in the faculty hall asking for participation in a research study. All these volunteers were assessed by the same trained clinician to ensure that they fulfilled all the inclusion and exclusion criteria that were applied in our group's previous publications [26, 41, 42] and previous Objectives 1 and 2. The inclusion criteria were the following: systemically healthy adult volunteers aged between 20 and 45 with a good oral health status, namely a minimum of 24 permanent teeth with no evidence of gingivitis or periodontitis (Community Periodontal Index score = 0) [43] and an absence of untreated caries at the start of the study. The following exclusion criteria were applied: smoker or former smoker, the presence of dental prostheses or orthodontic devices, antibiotic treatment or routine use of oral antiseptics in the previous 3 months, and the presence of any systemic disease that could alter the production or composition of saliva. Before the start of each test or experiment, a full mouth scaling with ultrasonic instruments and teeth polishing with a rubber cup after dental disclosure were performed by the same trained clinician on all the selected participants (Figure 3.1). Written informed consent was obtained from all the volunteers. To achieve the aims of the study, all the participants performed two different tests.

To test the antibacterial activity of the two EO solutions, an *in situ* model of PL-biofilm growth was used. An individualised thermoplastic appliance called intraoral disk-holding splints (IDODS) as we used in previous Objectives 1 and 2 with capacity to hold a total of six glass disks was made for each of the volunteers.

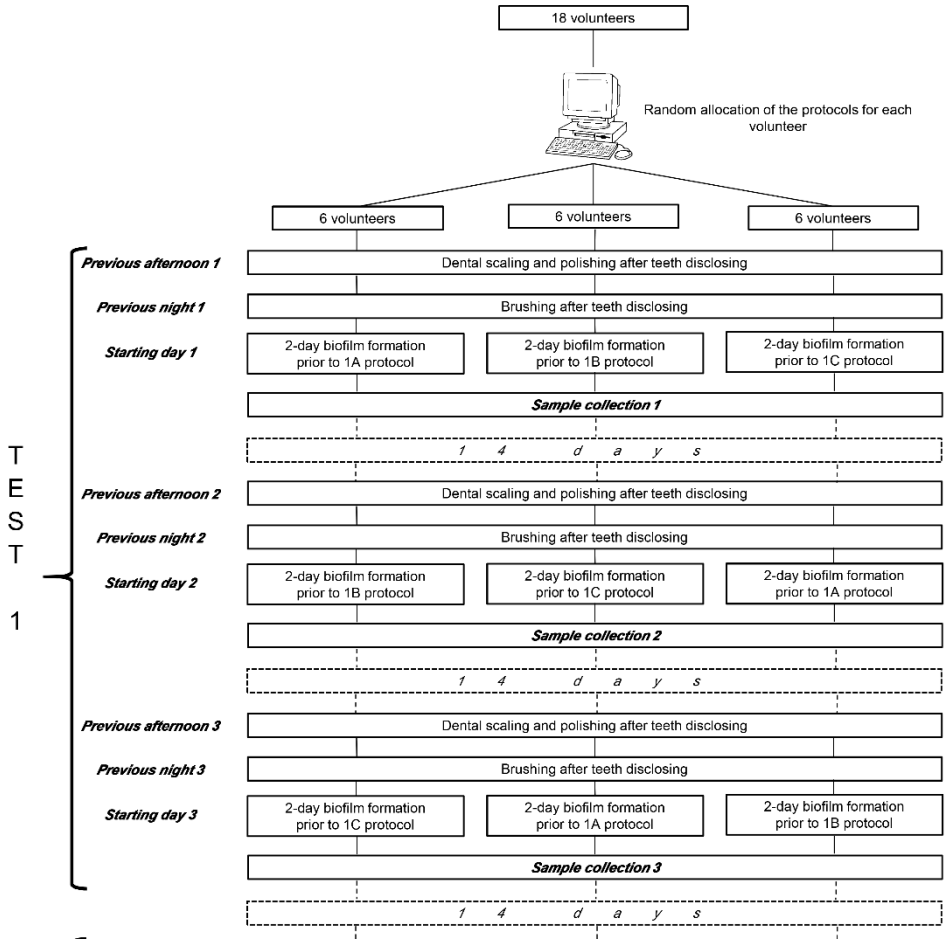


Figure 3.1. Protocol of the study (continues on the next page).

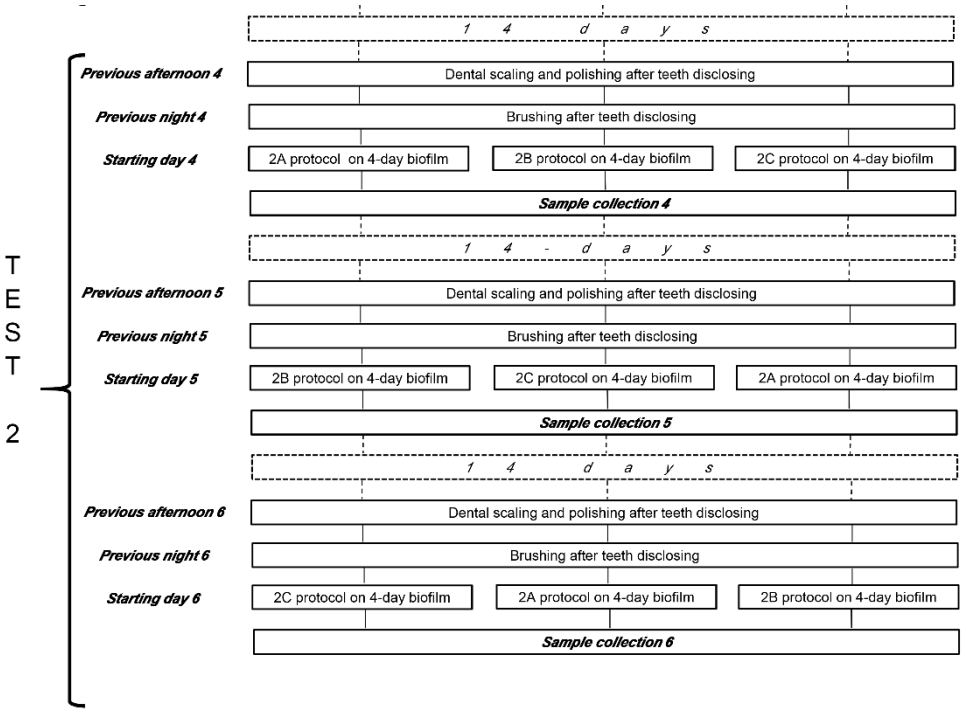


Figure 3.1. Protocol of the study (continuation)

3.3.1. TEST 1: PROTOCOL OF THE STUDY

The first experiment consisted of evaluating the immediate antibacterial effect and substantivity of the EO and Af-EO solutions. The volunteers wore an IDODS for 48 hours to enable growth of the PL-biofilm. They were allowed to remove it from the oral cavity only during meals and to perform oral hygiene measures. Volunteers were told to impregnate a sterile gauze with 5 mL of saline (the gauzes and the saline were provided as well) and extend it on the base of the plastic box, support the splints on it and close the box, leaving them at room temperature. The maximum allowed time that the volunteers had to eat and perform the oral hygiene measures were 20 minutes. They were advised to do just three meals per day avoiding eating or drinking in-between. The only drink permitted during meals was still

water. In order to not disturb the formation of the PL-biofilm, the volunteers could not use any toothpaste or mouthwash as a complement to the mechanical removal of bacterial plaque.

After 48 hours, the glass disks were withdrawn one by one from the splint from each volunteer (from right to left in a distal-mesial direction) at baseline, 30 seconds, and 1, 3, 5, 7 hours after performing the following mouthwashes under the supervision and with the IDODS present in the oral cavity:

1A) A single, 30-second mouthwash with 20 mL of sterile water (negative control) (M-WATER).

-OR

1B) A single, 30-second mouthwash with 20 mL of a traditional EO formulation (Listerine® Mentol™, Listerine®, Johnson & Johnson, Madrid, Spain) (M-T-EO).

-OR

1C) A single, 30-second mouthwash with 20 mL of an alcohol-free EO solution (Listerine® ZERO™, Listerine®, Johnson & Johnson, Madrid, Spain) (M-Af-EO).

On the day of the experiment, the volunteers were not allowed to eat or drink during the tests. A collection of the different PL-biofilm samples started at 11:50 AM (baseline sample) and finished at 7:00 PM (the final sample was obtained 7 hours after using the mouthwash).

Using an internet-based, balanced randomisation system [44], which indicated the mouthwash that each subject should use first, second and third, all the volunteers used the three mouthwashes, with a rest period of

2 weeks between each test (Figure 3.1). Although they were not told the type of mouthwash they were going to use, the obvious differences in taste between the three mouthwashes made allocation concealment to the volunteer impossible. The antiseptics/control were prepared in opaque bottles labelled with an A, B, or C depending on the containing solution.

3.3.2. TEST 2: PROTOCOL OF THE STUDY

The second experiment consisted of evaluating the antiplaque effect of both EO formulas. During the 4 days (96 hours) of duration of each mouthwash protocol of the present study, each volunteer wore the splints with the glass disks, withdrawing them from the oral cavity only during meals and to perform oral hygiene procedures, using only the mechanical removal of bacterial plaque with water, without the use of any toothpaste or mouthwash. They were advised to do just three meals per day avoiding eating or drinking in-between. The only drink permitted during meals was still water. While eating and brushing their teeth, the splints were stored in a provided opaque plastic box (the type used to store removable orthodontic devices). Volunteers were told to impregnate a sterile gauze with 5 mL of saline (the gauzes and the saline were provided as well) and extend it on the base of the plastic box, support the splints on it and close the box, leaving them at room temperature. The maximum allowed time that the volunteers had to eat and perform the oral hygiene measures were 20 minutes.

Using the permitted mechanical oral hygiene measures (without the IDODS), the volunteers performed the following protocols based on the manufacturers' instructions (with the IDODS in the oral cavity) over 4 days in the morning (8.30) after breakfast and at night (22.00) after dinner:

2A) A 30-second mouthwash with 20 mL of sterile water (negative control) (4D-WATER).

-OR

2B) A 30-second mouthwash with 20 mL of a traditional EO formulation (Listerine® Mentol™, Listerine®, Johnson & Johnson, Madrid, Spain) (4D-T-EO).

-OR

2C) A 30-second mouthwash with 20 mL of an alcohol-free EO solution (Listerine® ZERO™, Listerine®, Johnson & Johnson, Madrid, Spain) (4D-Af-EO).

The collection of the samples was carried out individually at 8 AM in the morning so that those of each volunteer were analysed on different days. It was determined that a minimum of 10 hours should have elapsed since the last use of the mouthwash the previous night.

In this test, mouthwashes carried out by the volunteers were not supervised, but they were instructed to use a measured volume of the allocated solution. Although they were not told the type of mouthwash they were going to use, the obvious differences in taste between the three mouthwashes made allocation concealment to the volunteer impossible. The antiseptics/control were prepared in opaque bottles labelled with an A, B, or C depending on the containing solution, with 10 mL more than the quantity needed for completing the whole series of mouthwashes; the bottles were weighed before giving them to the volunteers. The day before the start of each experiment, after the full mouth dental scaling and polishing, participants were given by a masked investigator the

corresponding previously made splints, the weighted allocated opaque bottle, a plastic glass and a sterile 20 mL serum syringe with the objective of being the more precise possible with the quantity of solution used for the mouthwash. The day of the sample analysis, they were asked to bring back the bottles to weight them again with the solution left. All volunteers performed the three rinsing protocols, with a rest period of 2 weeks between each test. To assess a subject's compliance with the rinsing protocol, the bottles containing the rinse were weighed before they were given to the volunteers.

3.3.3. PROCESSING OF THE SAMPLES OF THE PL-BIOFILM

As the glass disks were removed from the splint, they were immediately immersed in 100 µL of a fluorescence solution of LIVE/DEAD® BacLight™ (Molecular Probes Inc., Leiden, The Netherlands) and kept in a dark chamber at room temperature for 15 minutes. Microscope observations were performed by a single investigator who was unaware of the study design using a Leica TCS SP2 laser scanning spectral confocal microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) with an HCX APOL 63x/0.9 water-immersion lens.

The selection of the representative fields of the samples, the capture of the data and the quantification of the PL-biofilm thickness was performed as previously presented in Objectives 1 and 2. The maximum biofilm thickness of each field was divided into three zones or equivalent layers: the outer layer (layer 1), the middle layer (layer 2) and the inner layer (layer 3).

The quantification of bacterial viability (BV) in the series of XY images was determined using a cytofluorographic analysis (Leica confocal

software). In this analysis, the images of each fluorochrome were defined as “channels” (SYTO 9 occupies the green channel and Propidium Iodide (PI) the red channel). To conduct the computations automatically, a Matlab toolbox called Dentius biofilm was developed. The main program reads all the images from an experiment, organised in a folder tree, with the image folder at the top, the experiment folder below this and all the patient folders at the bottom. The program automatically computes the number of disks, fields and 1 µm sections from the images stored in each patient's folder.

The program considers the parameters fixed by experts: the BV is characterised by a high value in the green channel (over 100, with a range between 0 and 255) and a low value in the red channel (below 100). Bacteria are considered not to be viable if the values are high in the red channel (over 100) and low in the green channel (below 100). Values that are high in both channels (over 100) are visually orange and are considered non-viable bacteria. The program counts the number of pixels under these conditions to compute the BV percentage for each 1 µm section (viable bacteria/ viable bacteria+ non-viable bacteria x 100). Determination of the mean BV percentage in each field required sections with a minimum biofilm area of 250 µm2 (approximately 4,750 pixels).

The program also considers the case where epithelial nuclei are present. These are characterised by red compact areas with a size that is greater than the bacteria, and these red points must not be counted as non-viable bacterial population. To eliminate these pixels, the program disregards epithelial cells, which are characterised by having a high value in the red channel, an area greater than 200 pixels, compact regions with a solidity greater than 0.7, and a minimum value of the mean intensity of 180. These parameters were fixed using a training set. With this methodology, there could be some misdetections, but the effect on the BV was very low,

as what was important was the elimination of vast areas with a high intensity.

All the results obtained for each section, field and disk from each patient were stored in a worksheet to be analysed by the researchers. The BV percentage was also stored before and after eliminating the epithelial nuclei and their properties to localise them over the image. The mean BV percentage of the biofilm was calculated for the corresponding sample and each biofilm layer.

In Test 2, apart from the thickness and BV, the covering grade (CG) was also assessed. This concept is the percentage of the surface substrate covered by the biofilm. The cytofluorogram itself was used for this purpose. From the maximum projection (superposition of all captured planes) of each of the analysed fields, the CG percentage was obtained by calculating the sum of the bacterial mass (viable and non-viable) with regard to the total surface of the field (% positive within the total area).

3.3.4. STATISTICAL ANALYSIS

The statistical analyses were performed using the R software [45]. The Shapiro-Wilk test was carried out to analyse the distribution of the quantitative variables associated with the PL-biofilm (thickness, CG and BV), showing mostly these microscopic parameters a non-normal distribution in both tests.

In Test 1 (substantivity) and Test 2 (antiplaque effect), the Friedman test was used for intra-mouthwash and inter-mouthwash comparisons using all the PL-biofilm samples (including differentiating between the three biofilm layers). In both tests, the Wilconxon test was used for pairwise comparisons (with Bonferroni adjustment) of the intra- and inter-mouthwash

results (including differentiating between the three biofilm layers). The significance level established was a *p* value less than 0.05. In Test 1, the Bonferroni-corrected *p* values applied were <0.003 and <0.016, and in Test 2, this value was <0.016.

3.4. RESULTS

A total of 30 volunteers were evaluated to achieve the calculated sample size (n = 19). When this number of participants meeting the inclusion and exclusion criteria was reached, the enrolment process was ended. A total of 11 subjects were ineligible as they did not satisfy all of the inclusion criteria. All the participants performed both tests, although a subject was excluded after performing the Test 1 for an unexpected event. No adverse effects were reported by them at any stage of the experiment. Eighteen subjects completed the rinsing regimens satisfactorily in both tests. In Test 2, the returns of each product suggested good compliance with the instructions. In relation to demographic characteristics of the selected participants, eight were females and ten males with a mean age of 23.3 ± 1.8 years. No adverse or side effects were observed by investigators or reported by the volunteers after the completion of any of the rising cycles.

3.4.1. TEST 1

- *Influence of a single mouthwash with traditional and alcohol-free essential oils on the thickness of the PL-biofilm*

Neither the T-EO nor the Af-EO antiseptics had the capacity to reduce the thickness of the PL-biofilm of 48 hours after a single application. Their baseline thicknesses were 21.81 ± 5.28 µm and 20.71 ± 4.13 µm, respectively. After a single mouthwash, the thicknesses were slightly

reduced (20.19 ± 3.62 µm and 18.58 ± 3.14 µm, respectively), but did not achieve statistical significance.

- *Influence of a single mouthwash of* traditional *and alcohol-free essential oils on the bacterial viability of the PL-biofilm*

The baseline BV ranged between 64% and 80% for all the three rising protocols, with no statistical differences between them. Both EO formulations achieved similar results at all the time points measured. In fact, no differences were found between them from the immediate sample (30 seconds) to the 7-hour sample (Figure 3.2). The EO formulations were effective at reducing the BV after a single mouthwash (BV at 30 seconds for M-T-EO and M-Af-EO = 6.53 ± 7.60% and 4.13 ± 3.89%, respectively; $p<0.001$). These results were statistically lower than those from the M-WATER (62.39 ± 8.17%; $p<0.001$). Both solutions were able to keep the BV under baseline levels for 7 hours (BV at 7 hours for M-T-EO and M-Af-EO = 18.20 ± 9.38% and 20.10 ± 10.27%, respectively; $p<0.001$). Again, these findings were statistically lower than those from the M-WATER (BV at 7 hours = 76.78 ± 4.40%; $p<0.001$) (Figures 3.2 and 3.3).

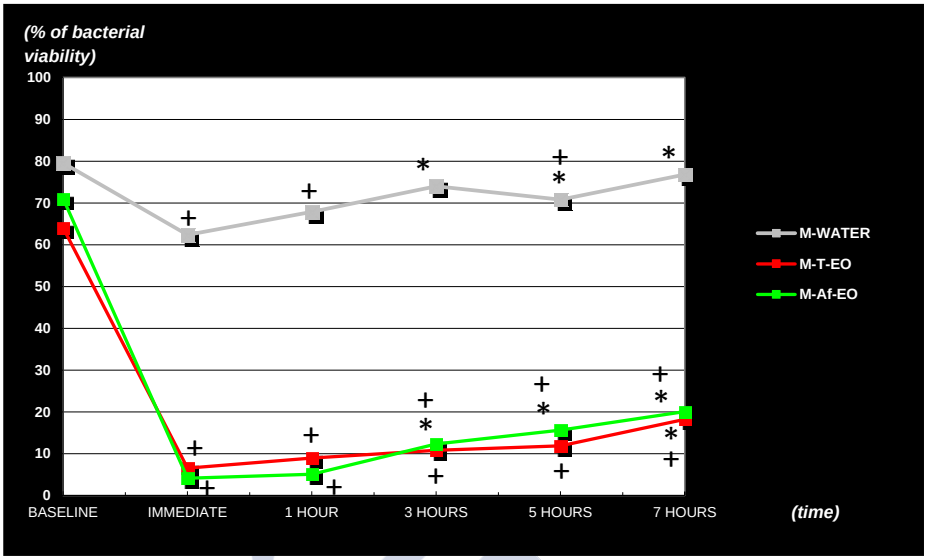
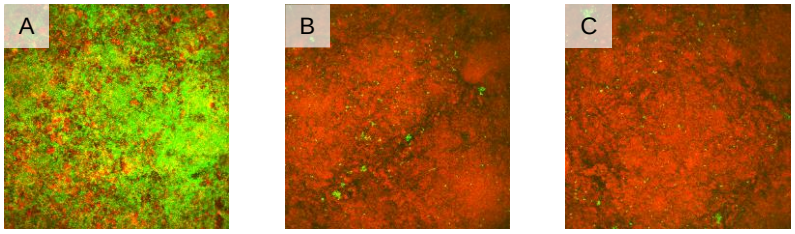
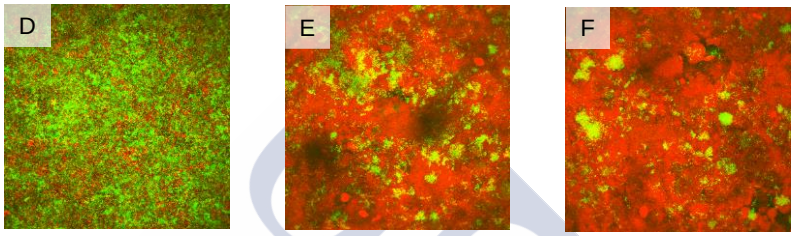


Figure 3.2. Percentages of the bacterial viability of the PL-biofilm in baseline conditions, at 30 seconds, 1, 3, 5 and 7 hours after a single mouthwash with sterile water (M-WATER), with essential oils with alcohol (M-T-EO) and with essential oils without alcohol (M-Af-EO). *Statistically significant differences in regard to the 30-second sample (p<0.003). $^{+}$Statistically significant differences in regard to the baseline sample (p<0.003).

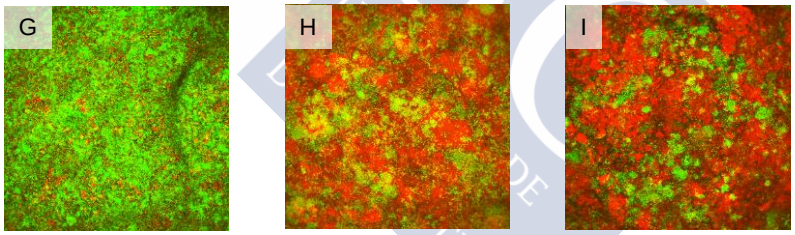
The BV recovery concerning the 30-second sample, significant recovery was not achieved until 7 hours after the use of an M-T-EO rinse (BV at 30 seconds *vs.* 7 hours for M-T-EO = 6.53 ± 7.60% *vs.* 18.20 ± 9.38%; p<0.001). However, for the M-Af-EO solution, significant recovery was identified in the 3-hour sample (BV at 30 seconds *vs.* 3 hours for M-Af-EO = 4.13 ± 3.89% *vs.* 12.35 ± 8.86%, p<0.001) (Figure 3.2).



A, B and C. Representation of the obtained bacterial viabilities 30 seconds after a single mouthwash with: A. Sterile water (M-WATER) (62.4%); B. Essential oils with alcohol (M-T-EO) (6.5%); C. Essential oils without alcohol (M-Af-EO) (4.1%).



D, E and F. Representation of the obtained bacterial viabilities 3 hours after a single mouthwash with: A. Sterile water (M-WATER) (67.9%); B. Essential oils with alcohol (M-T-EO) (10.8%); C. Essential oils without alcohol (M-Af-EO) (12.4%).



G, H and I. Representation of the obtained bacterial viabilities 7 hours after a single mouthwash with: A. Sterile water (M-WATER) (76.8%); B. Essential oils with alcohol (M-T-EO) (18.2%); C. Essential oils without alcohol (M-Af-EO) (20.1%).

Figure 3.3. Representative images of the obtained bacterial viabilities at 30 seconds, 3 hours and 7 hours after a single mouthwash with sterile water (M-WATER), essential oils with alcohol (M-T-EO), and essential oils without alcohol (M-Af-EO).

When it comes to differentiating between the three biofilm layers, the two EO antiseptics had lower BV levels in all the layers (Table 3.1). No significant differences were found in BV for the same biofilm layer between the EO formulations, with the outer layers being generally more viable than

the inner ones in all the samples. There were no significant differences between the three layers for the M-T-EO rinse (BV at 30 seconds by layers 1, 2 and 3 = 6.67 ± 6.80% *vs.* 5.63 ± 8.15% *vs.* 7.29 ± 9.17%, respectively; $p>0.016$) or between the deeper layers for the M-Af-EO rinse (BV at 30 seconds by layers 2 and 3 = 3.22 ± 3.24% *vs.* 3.51 ± 4.94%, respectively; $p>0.016$) (Table 3.1).

Table 3.1. Mean percentages of bacterial viability in PL-biofilm under basal conditions and the biofilm samples collected at 30 seconds and 1, 3, 5, and 7 hours after a single mouthrinse with sterile water (M-WATER), traditional essential oils solution (M-T-EO) and alcohol-free essential oils solution (M-Af-EO). Differences between the three biofilm layers, as well as intra-mouthrinse and inter-mouthrinse comparisons.

PL-biofilm BACTERIAL VIABILITY Mean ± Standard Deviation (%) Median (Interquartile Range)						
	BASAL	**30 SEC**	**1 H**	**3 H**	**5 H**	**7 H**
M-WATER						
Layer 1	86.82 ± 3.57 87.51 (2.18)	81.86 ± 6.63 80.48 (8.15)	85.46 ± 5.78 83.31 (6.89)	89.11 ± 6.13 91.90 (8.74)	86.26 ± 3.76 87.36 (6.29)	90.75 ± 3.45 89.54 (4.58)
Layer 2	82.06 ± 5.02 80.48 (3.87)	69.21 ± 7.83 68.53 (8.15)	73.94 ± 9.79 75.54 (6.02)	82.75 ± 5.98 80.91 (8.06)	77.20 ± 8.07 78.34 (11.84)	83.07 ± 4.25 84.73 (5.48)
Layer 3	69.74 ± 16.21 73.69 (22.91)	36.08 ± 20.97 31.09 (26.52)	44.18 ± 14.80 44.34 (30.87)	50.12 ± 13.22 56.27 (15.80)	48.95 ± 17.78 52.45 (32.48)	56.53 ± 12.59 60.34 (9.10)
M-T-EO						
Layer 1	75.54 ± 17.28 75.53 (19.71)	6.67 ± 6.80 4.44 (6.06)	9.90 ± 17.56 4.16 (4.80)	18.49 ± 14.37 16.36 (20.45)	22.35 ± 14.73 15.56 (28.93)	35.42 ± 19.00 35.55 (31.05)
Layer 2	67.86 ± 21.95 75.91 (32.06)	5.63 ± 8.15 2.77 (3.53)	7.97 ± 18.16 1.69 (4.65)	8.04 ± 10.79 3.68 (7.61)	8.58 ± 7.78 5.45 (6.14)	13.26 ± 9.09 9.91 (11.96)
Layer 3	48.56 ± 28.37 51.87 (32.67)	7.29 ± 9.17 1.72 (12.59)	9.02 ± 17.95 1.35 (6.06)	5.81 ± 9.26 0.52 (7.15)	4.76 ± 7.62 1.37 (4.29)	5.92 ± 9.21 2.56 (5.05)
M-Af-EO						
Layer 1	84.74 ± 15.33 90.03 (9.74)	5.66 ± 5.72 3.88 (6.75)	10.01 ± 8.30 8.82 (10.24)	20.74 ± 14.73 18.56 (12.87)	27.69 ± 18.15 21.85 (21.27)	41.99 ± 18.96 46.53 (27.42)
Layer 2	76.37 ± 19.38 81.93 (27.11)	3.22 ± 3.24 1.76 (5.33)	4.04 ± 4.25 2.46 (4.84)	9.70 ± 8.52 7.62 (10.00)	12.67 ± 10.22 8.32 (14.72)	14.28 ± 11.27 11.35 (14.96)
Layer 3	51.95 ± 31.30 59.06 (56.86)	3.51 ± 4.94 2.01 (3.13)	1.23 ± 1.18 0.98 (1.30)	6.63 ± 8.39 4.23 (7.16)	6.53 ± 7.06 3.38 (8.52)	4.02 ± 6.50 1.50 (4.27)
INTRA-MOUTHWASH ANALYSIS						

Statistical significance						
	BASAL	**30 SEC**	**1 H**	**3 H**	**5 H**	**7 H**
M-WATER						
Layer 1 *vs.* Layer 2	*p*<0.016	*p*<0.016	*p*<0.016	---	*p*<0.016	*p*<0.016
Layer 1 *vs.* Layer 3	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016
Layer 2 *vs.* Layer 3	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016
M-T-EO						
Layer 1 *vs.* Layer 2	*p*<0.016	---	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016
Layer 1 *vs.* Layer 3	*p*<0.016	---	---	*p*<0.016	*p*<0.016	*p*<0.016
Layer 2 *vs.* Layer 3	*p*<0.016	---	---	*p*<0.016	---	*p*<0.016
M-Af-EO						
Layer 1 *vs.* Layer 2	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016
Layer 1 *vs.* Layer 3	*p*<0.016	---	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016
Layer 2 *vs.* Layer 3	*p*<0.016	---	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016

INTER-MOUTHWASH ANALYSIS Statistical significance						
	BASAL	**30 SEC**	**1 H**	**3 H**	**5 H**	**7 H**
M-WATER *vs.* M-T-EO						
Layer 1 *vs.* Layer 1	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016
Layer 2 *vs.* Layer 2	---	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016
Layer 3 *vs.* Layer 3	---	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016
M-WATER *vs.* M-Af-EO						
Layer 1 *vs.* Layer 1	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016
Layer 2 *vs.* Layer 2	---	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016
Layer 3 *vs.* Layer 3	---	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016	*p*<0.016
M-Af-EO *vs.* M-T-EO						
Layer 1 *vs.* Layer 1	---	---	---	---	---	---
Layer 2 *vs.* Layer 2	---	---	---	---	---	---
Layer 3 *vs.* Layer 3	---	---	---	---	---	---

---- Not a statistically significant difference.

M-WATER = a single, 30-second mouthwash with 20 mL of sterile water; M-T-EO = A single, 30-second mouthwash with 20 mL of a traditional essential oils solution; M-Af-EO = A single, 30-second mouthwash with 20 mL of an alcohol-free essential oils solution.

3.4.2. TEST 2

- *Influence of a 4-day protocol of traditional and alcohol-free mouthwashes on the thickness and covering grade of the PL-biofilm*

The Af-EO rinses were more effective than the T-EO formulation at reducing the thickness of the oral biofilm after 4 days of use (thickness for 4D-T-EO *vs.* 4D-Af-EO = 9.92 ± 2.87 µm *vs.* 7.9 ± 2.91 µm; $p = 0.012$), but

both solutions were more powerful than the negative control (thickness for 4D-WATER = 22.76 ± 6.21 µm; p<0.001) (Table 3.2 and Figure 3.4).

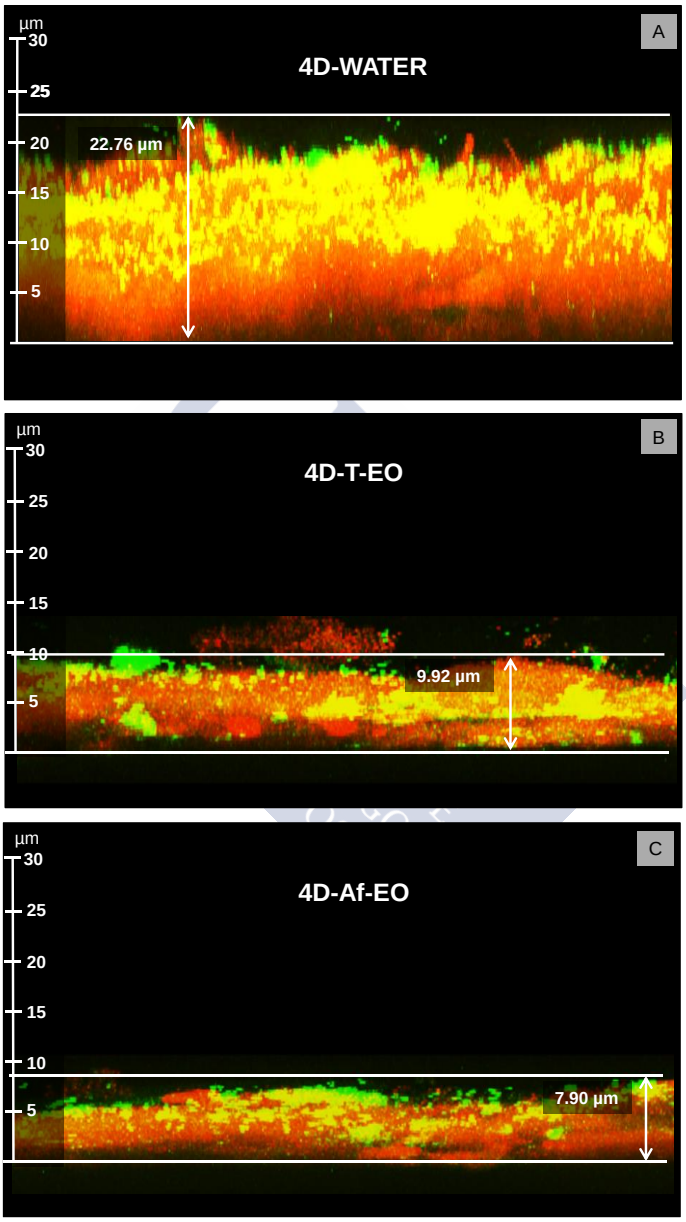


Figure 3.4. Graphical representation of the obtained thickness after 4 days of using twice daily: A) sterile water (4D-WATER); B) traditional solution of essential oils (4D-T-EO) and C. alcohol-free essential oils solution (4D-Af-EO).

The Af-EO rinse was more efficient than the T-EO solution at reducing the CG of the oral biofilm after 4 days of use (CG for 4D-T-EO *vs.* 4D-Af-EO = 46.61 ± 19.12% *vs.* 33.36 ± 12.01%, respectively; $p = 0.001$). The two EO solutions were significantly more effective than the negative control at reducing the CG (CG for 4D-WATER = 73.92 ± 17.49%; $p<0.001$) (Table 3.2 and Figure 3.5).

Table 3.2. Mean values of thickness, covering grade and bacterial viability, of the oral biofilm after 4 days of applying the three different protocols.

BACTERIAL VIABILITY, THICKNESS AND COVERING GRADE OF THE PL-BIOFILM (%) Mean ± Standard Deviation (%) Median (Interquartile Range)			
	THICKNESS	**COVERING GRADE**	**BACTERIAL VIABILITY**
4D-WATER	22.76 ± 6.21 24.24 (5.72)	73.92 ± 17.49 76.23 (15.59)	51.35 ± 5.38 50.70 (6.69)
4D-T-EO	9.92 ± 2.87* 9.66 (3.80)*	46.61 ± 19.09* 45.49 (24.51)*	26.27 ± 14.61* 22.68 (15.87)*
4D-Af-EO	7.90 ± 2.91*§ 7.23 (2.29)* §	33.36 ± 12.01*§ 32.97 (14.92)* §	31.08 ± 16.52* 29.66 (14.61)*

4D-WATER = period of 4 days while the volunteer performs two daily mouthwashes with 20 mL of sterile water; 4D-T-EO = period of 4 days while the volunteer performs two daily mouthwashes with 20 mL of a traditional essential oils solution; 4D-Af-EO = period of 4 days while the volunteer performs two daily mouthwashes with 20 mL of a alcohol free-essential oils solution. *Statistically significant differences in regard to the 4D-WATER, $p<0.016$. §Statistically significant differences in regard to the 4D-T-EO, $p<0.016$.

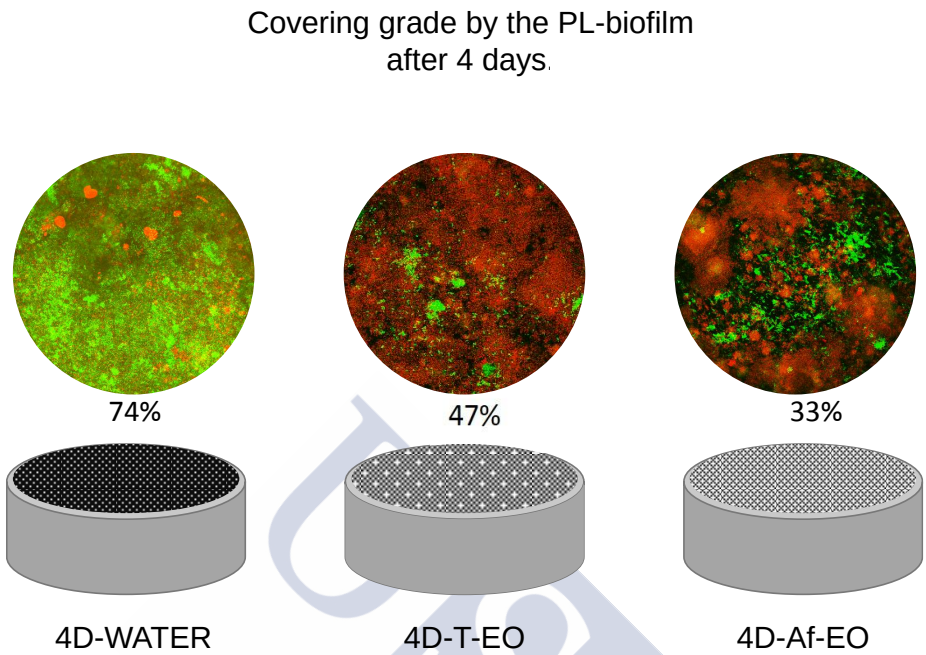


Figure 3.5. Representative images and graphics of the disks covering grade by the plaque-like biofilm after 4 days of continuous use of sterile water (4D-WATER), traditional essential oils (4D-T-EO) and alcohol-free essential oils (4D-Af-EO).

- *Influence of a 4-day protocol of traditional and alcohol-free essential oils mouthwashes on the bacterial viability of the PL-biofilm*

The T-EO and Af-EO formulations after 4 days of use were effective at maintaining the BV at significantly lower levels than the negative control (BV for 4D-T-EO and 4D-Af-EO *vs*. 4D-WATER = 26.27 ± 14.61% and 31.08 ± 16.52% *vs.* 51.35 ± 5.38%, respectively; $p<0.001$). The T-EO rinse showed slightly higher bactericidal activity than the Af-EO solution, although no significant results were achieved (Table 3.2). Regarding the BV by layers, the outer layers had significantly more BV than the inner ones in all the experiments. The T-EO and Af-EO formulations were significantly more effective at reducing the BV of layers 1 and 2 than the negative control, with layer 3 unaltered (BV for layer 1 = 40.10 ± 17.31% and 39.81 ± 19.09% *vs*.

82.47 ± 7.58%; BV for layer 2 = 24.32 ± 16.16% and 30.73 ± 17.06% *vs*. 51.76 ± 13.53%; $p<0.001$ for all comparisons) (Table 3.3).

Table 3.3. Mean percentages of the bacterial viability of the oral biofilm after 4 days of applying the three different protocols, differentiating by layers.

PL-biofilm BACTERIAL VIABILITY BY LAYERS Mean ± Standard Deviation (%) Median (Interquartile Range)			
	LAYER 1	**LAYER 2**	**LAYER 3**
4D-WATER	82.47 ± 7.58[ǂ] 81.00 (11.38) [ǂ]	51.76 ± 13.53[ǂ] 45.30 (16.82) [ǂ]	19.83 ± 12.60[ǂ] 17.42 (8.19) [ǂ]
4D-T-EO	40.10 ± 17.31*[ǂ] 39.52 (21.20)* [ǂ]	24.32 ± 16.16*[ǂ] 21.17 (15.98)* [ǂ]	14.40 ± 14.34[ǂ] 11.00 (17.00) [ǂ]
4D-Af-EO	39.81 ± 19.09*[ǂ] 35.14 (22.21)* [ǂ]	30.73 ± 17.06*[ǂ] 29.26 (14.16)* [ǂ]	22.71 ± 17.05[ǂ] 17.37 (12.04) [ǂ]

4D-WATER = period of 4 days while the volunteer performs two daily mouthwashes with 20 mL of sterile water; 4D-T-EO = period of 4 days while the volunteer performs two daily mouthwashes with 20 mL of a traditional essential oils solution; 4D-Af-EO = period of 4 days while the volunteer performs two daily mouthwashes with 20 mL of an alcohol-free essential oils solution. *Statistically significant differences in regard with the 4D-WATER between the same layers (inter-mouthwash and intra-layer comparisons), $p<0.016$. [ǂ]Statistically significant differences between the different layers of the same mouthwash (intra-mouthwash and inter-layer comparisons, $p<0.016$.

3.5. DISCUSSION

The present series is the first study to compare the antibacterial activity *in vivo* that T-EO and Af-EO solutions have on the undisturbed oral biofilm. From a methodological perspective, to perform all the BV computations automatically through a Matlab toolbox called Dentius biofilm ensures that the experiment's findings are accurate, quick to obtain, reliable and repeatable, which is very important when it comes to comparing results and extracting robust conclusions.

3.5.1. INFLUENCE OF A SINGLE MOUTHWASH OF TRADITIONAL AND ALCOHOL-FREE ESSENTIAL OILS ON THE THICKNESS OF THE PL-BIOFILM

In the literature, very variable thickness measures have been identified in a non-disturbed 48-hour-biofilm [26, 31, 33, 41, 42, 46-51]. This is due to the variability in the plaque formation of the different volunteers [47] or, in some cases, the way the thickness is measured by the authors [42]. In the present study, the 48 hour-biofilm had a thickness of 20 µm-22 µm, which agrees with those found in studies on the PL-biofilm formed *in situ* (21-27 µm) [26, 33, 42]. After a single mouthwash, no reduction in the PL-biofilm thickness was found with any of the EO solutions. This finding is consistent with the previous literature regarding EO [33]; it was only in the case of the 0.2% CHX solution that some slight reductions could be detected [26].

3.5.2. INFLUENCE OF A SINGLE MOUTHWASH OF TRADITIONAL AND ALCOHOL-FREE ESSENTIAL OILS ON THE BACTERIAL VIABILITY OF THE PL-BIOFILM

In the present series, both EO solutions achieved excellent antibacterial activity, with BV reductions between 57% and 67% and reaching levels of around 5% at 30 seconds. After the very high immediate antibacterial effect that both formulations had, the BV started its slow recovery, albeit faster in the case of the T-EO solution. In any case, both EO mouthwashes were able to maintain the BV under basal levels until 7 hours after a single application, keeping at that moment a 45-50% difference in BV compared to the baseline values.

There are no results in relation to the immediate effect or substantivity for the Af-EO rinse, but some research has been conducted in this field on

T-EO solutions. In a previous study of our research group, in which we compared the antibacterial activity of T-EO with that of 0.2% CHX, we detected that the T-EO antiseptic had an even greater antibacterial effect than the CHX (Objective 1). In fact, the T-EO rinse had an immediate BV near to zero (it was around 1%) compared to 5% for the 0.2% CHX. In that study, the T-EO rinse also maintained the BV under baseline levels up to 7 hours post-rinsing (Objective 1). In contrast, other studies found that T-EO solutions were not as effective as described in this paper. In this sense, Gosau et al. [32] observed that the BV after a single application of T-EO was around 20%. However, a methodological comment should be made at this point. Gosau et al. used a similar *in vivo* model to the one used in our paper, but the two studies differ in how the T-EO rinses were applied [32]. In our experiment, the T-EO were applied as an active mouthwash by the volunteers; in Gosau et al.'s study the disks were removed from the oral cavity and immersed passively in a T-EO solution. This procedure has been found not to be as effective as an active mouthwash at reducing the BV of the PL-biofilm formed *in situ*, particularly in its deepest regions [41]. Furthermore, differences have been found between not only EO rinses but also 0.2% CHX solutions [41]. For these reasons, the application methodology of an oral antiseptic should be taken into account when considering the *in situ* antibacterial activity, since oral hydrodynamic forces play a major role in the activation and penetration of the antiseptic in the complex bacterial network that an oral biofilm constitutes [41].

The PL-biofilm obtained after 2 days followed a previously described pattern [33, 42], with a BV that was significantly lower in the deeper than the outer layers. This pattern was also followed after the different mouthwashes were applied, except in the immediate samples. After that,

the BV gradually increased mainly in the outer layers of the biofilm. These results are also consistent with previous research in the field [26].

3.5.3. INFLUENCE OF A 4-DAY PROTOCOL OF TRADITIONAL AND ALCOHOL-FREE ESSENTIAL OIL MOUTHWASHES ON THE THICKNESS AND COVERING GRADE OF THE PL-BIOFILM

Despite the multitude of studies conducted on a 4-day model of plaque regrowth [36, 37, 42, 52-56], very few of them have analysed the plaque without distortion [42]. This fact is not a minor issue, as it has been proved that the 3-D structure of the oral biofilm plays a crucial role in the biofilm's defence against external agents and is essential in its development [30]. Also, distorting the original structure of the oral biofilm does not permit the measurement of the dental plaque thickness, or its BV by layers when it comes to assessing the penetration power of the antiseptic [42].

After 4 days of dental plaque accumulation with the performance of daily mouthwashes with sterile water, the obtained thickness (22.76 µm) was consistent with that described in previous publications using the same methodological design (Objective 2) (which achieved an oral biofilm thickness of 23.43 µm). In another study carried out by the Arweiler's group [57] obtained a biofilm of 25.33 µm after immersing the oral biofilm samples in saline twice daily for 5 days. These thicknesses are in line with those obtained by other experiments assessing periods of 3-5 days of evolution [46, 48, 58] when studying the oral biofilm, and varied between 7 µm and 45 µm.

The two EO mouthwashes were effective at reducing the thickness of the biofilm formed after 4 days compared to the negative control. In fact, the mean thickness measured for the Af-EO solution was almost three times less thick than that from the sterile water (7.9 µm *vs.* 22.8 µm); in the case

of the EO solution, the obtained mean thickness was less than half that for the sterile water (9.9 µm *vs.* 22.8 µm). These results are in accordance with previous investigations on T-EO and other antiseptics [59, 60]. In an earlier study, researchers found a thickness of 10 µm after 4 days of the continuous use of T-EO (Objective 2). Jentsch et al. [60], using a scanning electron microscope (SEM), obtained a thickness of 10.5 µm after 3 days of the daily use of T-EO. For other antiseptics, the findings vary depending on the mouthwash and the duration of the experiment. For 0.2% CHX, the results ranged from 6.5 after 4 days (Objective 2) to 11.91 after 5 days [57]. When a lower concentration was applied (0.12% CHX), the thickness rose to 14.02 µm after 3 days [59]; in this same study, the antiplaque effect of the stannous fluoride was evaluated, obtaining a thickness of 11.9 µm after the same period of time.

After comparing the thickness obtained for both EO formulations, there was an unexpected result: the Af-EO rinse was more effective than the T-EO rinse at reducing the biofilm's thickness. This result will be discussed further, along with the CG results.

The CG can be predictive of the adaptation of microorganisms to environmental influences [61]. For this reason, this parameter is crucial and is directly related to the antiplaque effect of an antiseptic agent. Despite this, it has traditionally been forgotten in microbiological studies. In fact, to the best of the author's knowledge, there is only one study on this issue involving a 4-day PL-biofilm *in situ* (Objective 2). Our results are in accordance with this previous investigation, as the CG for the negative control was almost the same (around 73%-75%) and it was slightly better for the T-EO rinse (47% *vs.* 54%). EO solutions were, however, less effective than the 0.2% CHX, which had a CG of 20% in the time same

period. No results were found for the Af-EO rinse, which in the present series had a CG of 33% and were more efficient than the T-EO solution at reducing this microscopic parameter.

3.5.4. INFLUENCE OF A 4-DAY PROTOCOL OF TRADITIONAL AND ALCOHOL-FREE ESSENTIAL OIL MOUTHWASHES ON THE BACTERIAL VIABILITY OF THE PL-BIOFILM

After 4 days of growth with any disturbing agent other than sterile water, the oral biofilm had a BV of 51%. This observation is consistent with previous research in the field, with results that are slightly over 50% for the BV [57]. In addition, the lowest viability was in the deepest layer, which had BV levels that did not differ too much from those detected after the application of the T-EO mouthwashes.

This phenomenon has been repeatedly described in the literature since bacteria located in the lower strata of the biofilm receive fewer nutrients, and so acquire an inactive metabolic state [46, 62]. Furthermore, it is deeply related to the greater thickness and density of the biofilm, which makes the correct flow of nutrients and oxygen more difficult to achieve in the deeper layers [63].

In the present series, the BV of the 4-day PL-biofilm was reduced by the T-EO and Af-EO solutions (49% and 40%, respectively compared to the negative control). No other studies have been found in relation to the Af-EO, but T-EO rinses have previously been shown to have a greater antiplaque effect in terms of reducing BV of PL-biofilm after 4 days of use, achieving reduction levels close to those of 0.2% CHX (74% *vs.* 77%) (Objective 2). In another *in vivo* study, Arweiler et al. [57] found that 0.2%

CHX reduced BV of biofilm by 62% after 5 days of use compared to the negative control.

3.5.5. CLINICAL STUDIES ON THE ANTIPLAQUE EFFECT OF ESSENTIAL OIL FORMULATIONS

In the present study, a T-EO rinse containing alcohol and an Af-EO rinse were used, enabling the effects of one to be compared with those of the other. This is the first study in the literature to compare the antiplaque effect of T-EO and Af-EO solutions in an *in situ* model of undisturbed PL-biofilm grown after 4 days, with the thickness, CG and BV analysed. However, some studies in the literature have evaluated the effects of both antiseptics at 3 to 4 days [34, 36-39]. In these studies, the authors compare the antiplaque effect assessed by clinical parameters [34, 36, 38, 39] or the efficacy of both solutions at reducing *S. mutans* levels [37]. Marchetti et al. [34] used a 3-day plaque growth model in which the area occupied by plaque was evaluated after performing two daily rinses for 1 minute with 20 mL of different EO solutions. T-EO rinses were shown to be more effective at reducing clinical indexes of dental plaque after 3 days (2.18 *vs.* 2.46). This same group also performed subsequently a similar study [38], in which the same Af-EO formulation obtained a similar result (plaque index = 2.45) that was less effective than 0.2% CHX (plaque index = 1.41). Pizzo et al. [36] assessed the antiplaque effect of both EO solutions using a plaque index in a 4-day experiment. Equally, they found that the Af-EO rinses were not effective at reducing plaque levels (no differences about the negative control). These clinical results do not agree with the microscopic findings obtained in the present series, as the thickness and the CG percentage of the PL-biofilm were statistically lower for the Af-EO mouthwash concerning the EO mouthwash. These differences can be explained by the fact that the

previous three studies used an alcohol-free formulation (Curasept Daycare, Curaden International AG, Kriens, Suisse) and another with alcohol (Listerine®, Johnson & Johnson, S. Palomba-Pomezia, Italy) from different manufacturers. In our opinion, this methodological aspect can significantly influence the findings obtained. In fact, recently, another study from Marchetti's group [39] compared the alcohol-free and the traditional formulations with ethanol from the same manufacturer (Johnson & Johnson). They found that both solutions were effective compared to the negative control (plaque index = 1.7 *vs.* 2.3), which confirms the importance of comparing formulations from the same manufacturer. In our case, we used two products from the same manufacturer (Johnson & Johnson, Madrid), which is better for comparison purposes, since the active principles may not be the only ones responsible for the antiplaque effect.

3.5.6. ANTIPLAQUE EFFECT OF ALCOHOL-FREE ESSENTIAL OILS: THE IMPORTANCE OF THE "INACTIVE" INGREDIENTS IN THE FORMULA

In the formulation of oral antiseptics, the active principles play a significant role in the activity against the oral biofilm. However, in the case of CHX, for example, it has been shown that different formulations with the same concentration of active ingredients produce different results regarding antimicrobial efficacy [64]. The differences in the present series to other findings in the literature may relate to the presence of sodium lauryl sulphate, which is used to dissolve EO [65].

The better microscopic results in the present series of the Af-EO mouthwash in relation to the CG and thickness can be explained by a dual theory. The composition of the two rinses differs, apart from the ethanol, in

that sodium lauryl sulphate is present in Listerine® Zero™, but not in Mentol™. This component has been shown to be effective at reducing BV [55, 66, 67] and plaque formation [68, 69]. Its antibacterial effect may be due to the formation of pores in bacterial membranes, which could increase membrane fluidity, reduce phospholipid chains in the membrane, increase the rotation movement of lipid molecules, and change the lateral distribution of proteins and membrane lipids [66]. Its effect on dental plaque may be due to a loss of high-density particles present in the cell matrix. Robinson et al. [69] explained that the removal of structural material by this detergent essentially affects high-density proteins and molecules. This could improve the penetrability of the antiseptic, resulting in a greater antiplaque effect. The existence of an inhibitory effect of sodium lauryl sulphate on glucosyl and fructosyl transferases has been identified. These are the enzymes responsible for the synthesis of exopolysaccharides in *S. mutans* [66]. This component has been shown to be especially effective against *S. mutans*, as it also reduces lactate formation by 33% [66]. In fact, Ulkur et al. [37] found that both formulations tested in our study had the same effect against these bacteria in a 4-day oral biofilm model. In the present series, the bactericidal effects of the sodium lauryl sulphate may not have manifested, because the minimum inhibitory concentration has not been sufficient to affect on BV, although it may have acted on the metabolic level of the bacteria.

As for the other differentiating element, namely ethanol, its effects on the biofilm were studied extensively in the 1990s. Accordingly, the bacteria present in biofilms have adapted physiologically and become more resistant to stress, including that induced by antimicrobial agents [12, 70]. A possible mechanism by which alcohol resistance above concentrations over 4% could occur is the induction of an adaptive stress response by bacteria [12,

71, 72]. In fact, in previous studies, an increase in plaque growth *in vivo* was described in a 4-day model after rinsing twice a day with 50% ethanol [73]. Sissons et al. [12] reported that alcohol concentrations between 20% and 30% initially produced a rapid inactivation of the bacteria present in the biofilm, but quickly lost its activity and a large resistant population remained unchanged.

In the same sense, long-term studies [74] appear in the literature comparing the use of EO containing ethanol to a negative control of water and its dissolution vehicle (alcohol at 26.9% and the rest of the excipients). While EO had a significant antiplaque effect after 9 months of continuous use, the dissolution vehicle produced a 7.3% increase in plaque levels after this period of application. This result reinforces the theory that the ethanol *per se* could cause an increase in plaque formation.

3.5.7. ESSENTIAL OILS FORMULATION: THE ALCOHOL CONTENT AS A MATTER OF CONTROVERSY

The ethanol content in the formulation of oral antiseptics and its possible involvement in the development of oral cancer is a constant subject of discussion among authors [18, 75-77].

Zamora-Pérez et al. [75] found that after 30 days of daily use of a mouthwash with ethanol in its formulation there was a greater presence of micronuclei and nuclear abnormalities in oral mucosal cells. However, Eliot et al. [77], despite finding a slight association between a history of periodontal disease and head and neck cancer, could not associate this increase with the use of a mouthwash containing ethanol. Also, in a prospective study of 6 months, Bagán et al. [18] did not identify any cytological changes in cells of the oral mucosa after the routine use of two

daily mouthwashes containing 26.9% ethanol in their formulation. After a meta-analysis of epidemiological studies on the issue, Gandini et al. [19] concluded that there was no association between the alcohol content of mouthwashes and the development of oral cancer. However, Lachenmeier [21] questioned the power that epidemiological studies might have to demonstrate a relationship between the alcohol-content mouthwashes and the risk of developing oral cancer, concluding that, probably, this possible association will never be confirmed.

In addition, the German Institute for Risk Assessment (Bundesinstitut für Risikoberwertung) concluded: "*The contribution of mouthwashes to exposure to acetaldehyde is 0.25 µg/kg and extremely low compared to food and alcoholic drinks*". As a consequence: "*Oral rinses are not expected to have a carcinogenic or preneoplastic effect if used as intended as a result of the short contact of ethanol with the oral mucosa*". On the other hand, we must also take into account profound differences between the pure ethanol used as a solvent in mouthwashes and the alcohol from alcoholic drinks that often contain nitrosamines, polycyclic hydrocarbons and other toxins [78].

In any case, there are authors who have stated that although the association has not been proved, it would be better to be cautious and not prescribe mouthwashes with ethanol to pediatric patients and patients with a particular susceptibility to oral cancer, such as those who are alcohol dependent and those with genetic deficiencies in relation to the metabolism of acetaldehyde [22-24].

3.6. CONCLUSION

In a 2-day *in situ* undisturbed plaque-like biofilm model, after a single mouthwash, both essential oil formulations had very high immediate antibacterial activity and a substantivity which lasted for at least 7 hours after application. In a 4-day *in situ* undisturbed plaque-like biofilm model, both essential oil formulations demonstrated a very good antiplaque effect, although the alcohol-free formula performed better at reducing the biofilm thickness and covering grade.

Consequently, the alcohol-free essential oils solution represents a reliable option as antibacterial and antiplaque agent for the control of oral biofilm.

3.7. REFERENCES

1. **Marsh, P.D. and Bradshaw, D.J.**, *Microbiological effects of new agents in dentifrices for plaque control.* Int Dent J, 1993. 43(4 Suppl 1): p. 399-406.

2. **ten Cate, J.M. and Marsh, P.D.**, *Procedures for establishing efficacy of antimicrobial agents for chemotherapeutic caries prevention.* J Dent Res, 1994. 73(3): p. 695-703.

3. **Newman, H.N.**, *Art and science, culture and dental research.* J Dent Res, 1996. 75(1): p. 526-7.

4. **McDonnell, G. and Russell, A.D.**, *Antiseptics and disinfectants: activity, action, and resistance.* Clin Microbiol Rev, 1999. 12(1): p. 147-79.

5. **von Ohle, C., Gieseke, A., Nistico, L., Decker, E.M., DeBeer, D., and Stoodley, P.**, *Real-time microsensor measurement of local metabolic activities in ex vivo dental biofilms exposed to sucrose and treated with chlorhexidine.* Appl Environ Microbiol, 2010. 76(7): p. 2326-34.

6. **Tomás, I., Cousido, M.C., Tomás, M., Limeres, J., García-Caballero, L., and Diz, P.**, *In vivo bactericidal effect of 0.2% chlorhexidine but not 0.12% on salivary obligate anaerobes.* Arch Oral Biol, 2008. 53(12): p. 1186-91.

7. **Van Strydonck, D.A., Slot, D.E., Van der Velden, U., and Van der Weijden, F.**, *Effect of a chlorhexidine mouthrinse on plaque, gingival inflammation and staining in gingivitis patients: a systematic review.* J Clin Periodontol, 2012. 39(11): p. 1042-55.

8. **Neely, A.L.**, *Essential oil mouthwash (EOMW) may be equivalent to chlorhexidine (CHX) for long-term control of gingival inflammation but CHX appears to perform better than EOMW in plaque control.* J Evid Based Dent Pract, 2012. 12(3 Suppl): p. 69-72.

9. **Van Leeuwen, M.P., Slot, D.E., and Van der Weijden, G.A.**, *Essential oils compared to chlorhexidine with respect to plaque and parameters of gingival inflammation: a systematic review.* J Periodontol, 2011. 82(2): p. 174-94.

10. **Lis-Balchin, M., Deans, S.G., and Eaglesham, E.**, *Relationship between bioactivity and chemical composition of commercial essential oils.* Flav Frag J, 1998. 13: p. 98-104.

11. **Fine, D.H.**, *Mouthrinses as adjuncts for plaque and gingivitis management. A status report for the American Journal of Dentistry.* Am J Dent, 1988. 1(6): p. 259-63.

12. **Sissons, C.H., Wong, L., and Cutress, T.W.**, *Inhibition by ethanol of the growth of biofilm and dispersed microcosm dental plaques.* Arch Oral Biol, 1996. 41(1): p. 27-34.

13. **Marchetti E, M.S., Marzo G, Civisca A, Di Pietro C, Marinelli G**, *Effect of an essential-oil containing mouthrinse on the reduction of plaque and interproximal inflammation in comparison with an ethanol solution.* Prev Assist Dent, 2009. 35(4): p. 123-127.

14. *Listerine Mentol Composition*. Available from: https://www.listerineprofessional.com/products/antiseptic-mouthwash-rinse/cool-mint#active-ingredients. Last access on 14/09/2017.

15. **Penugonda, B., Settembrini, L., Scherer, W., Hittelman, E., and Strassler, H.**, *Alcohol-containing mouthwashes: effect on composite hardness.* J Clin Dent, 1994. 5(2): p. 60-2.

16. **Smigel, K.**, *High-alcohol mouthwashes are under scrutiny.* J Natl Cancer Inst, 1991. 83(11): p. 751.

17. **Llewelyn, J.**, *Oral squamous cell carcinoma. Mouthwashes may increase risk.* BMJ, 1994. 308(6942): p. 1508.

18. **Bagán, J.V., Vera-Sempere, F., Marzal, C., Pellin-Carcelen, A., Marti-Bonmati, E., and Bagán, L.**, *Cytological changes in the oral mucosa after use of a mouth rinse with alcohol. A prospective double blind control study.* Med Oral Patol Oral Cir Bucal, 2012. 17(6): p. e956-61.

19. **Gandini, S., Negri, E., Boffetta, P., La Vecchia, C., and Boyle, P.**, *Mouthwash and oral cancer risk quantitative meta-analysis of epidemiologic studies.* Ann Agric Environ Med, 2012. 19(2): p. 173-80.

20. **Moazzez, R., Thompson, H., Palmer, R.M., Wilson, R.F., Proctor, G.B., and Wade, W.G.**, *Effect of rinsing with ethanol-containing mouthrinses on the production of salivary acetaldehyde.* Eur J Oral Sci, 2011. 119(6): p. 441-6.

21. **Lachenmeier, D.W.**, *Alcohol-containing mouthwash and oral cancer--can epidemiology prove the absence of risk?* Ann Agric Environ Med, 2012. 19(3): p. 609-10.

22. **McCullough, M.J. and Farah, C.S.**, *The role of alcohol in oral carcinogenesis with particular reference to alcohol-containing mouthwashes.* Aust Dent J, 2008. 53(4): p. 302-5.

23. **La Vecchia, C.**, *Mouthwash and oral cancer risk: an update.* Oral Oncol, 2009. 45(3): p. 198-200.

24. **Vlachojannis, C., Winsauer, H., and Chrubasik, S.**, *Effectiveness and safety of a mouthwash containing essential oil ingredients.* Phytother Res: PTR, 2013. 27(5): p. 685-91.

25. **Manau Navarro, C. and Guasch Serra, S.**, *Métodos de control de la placa bacteriana*, in *Odontología preventiva y comunitaria: Principios, métodos y aplicaciones*, E. Cuenca Sala, C. Manau Navarro, and L. Serra Majem, Editors. 2003: Barcelona. p. 79.

26. **García-Caballero, L., Quintas, V., Prada-López, I., Seoane, J., Donos, N., and Tomás, I.**, *Chlorhexidine Substantivity on Salivary Flora and Plaque-Like Biofilm: An In Situ Model.* PLoS One, 2013. 8(12): p. e83522.

27. **Council on Dental Therapeutics**. *Guidelines for acceptance of chemotherapeutic products for the control of supragingival dental plaque and gingivitis.* J Am Dent Assoc, 1986. 112(4): p. 529-32.

28. **Singh, A., Daing, A., and Dixit, J.**, *The effect of herbal, essential oil and chlorhexidine mouthrinse on de novo plaque formation.* Int J Dent Hyg, 2013. 11(1): p. 48-52.

29. **Beyth, N., Yudovin-Farber, I., Perez-Davidi, M., Domb, A.J., and Weiss, E.I.**, *Polyethyleneimine nanoparticles incorporated into resin composite cause cell death and trigger biofilm stress in vivo.* Proc Natl Acad Sci U S A, 2010. 107(51): p. 22038-43.

30. **Wood, S.R., Kirkham, J., Marsh, P.D., Shore, R.C., Nattress, B., and Robinson, C.**, *Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy.* J Dent Res, 2000. 79(1): p. 21-7.

31. **Gu, H., Fan, D., Gao, J., Zou, W., Peng, Z., Zhao, Z., Ling, J., and LeGeros, R.Z.**, *Effect of ZnCl2 on plaque growth and biofilm vitality.* Arch Oral Biol, 2012. 57(4): p. 369-75.

32. **Gosau, M., Hahnel, S., Schwarz, F., Gerlach, T., Reichert, T.E., and Burgers, R.**, *Effect of six different peri-implantitis disinfection methods on in vivo human oral biofilm.* Clin Oral Implants Res, 2010. 21(8): p. 866-72.

33. **Dong, W.L., Zhou, Y.H., Li, C.Z., Liu, H., Shang, S.H., and Pan, B.Q.**, *[Establishment and application of an intact natural model of human dental plaque biofilm].* Shanghai Kou Qiang Yi Xue, 2010. 19(2): p. 196-201.

34. **Marchetti, E., Mummolo, S., Di Mattia, J., Casalena, F., Di Martino, S., Mattei, A., and Marzo, G.**, *Efficacy of essential oil mouthwash with and without alcohol: a 3-day plaque accumulation model.* Trials, 2011. 12: p. 262.

35. **Charles, C.A., Amini, P., Gallob, J., Shang, H., McGuire, J.A., and Costa, R.**, *Antiplaque and antigingivitis efficacy of an alcohol-free essential-oil containing mouthrinse: a 2-week clinical trial.* Am J Dent, 2012. 25(4): p. 195-8.

36. **Pizzo, G., Compilato, D., Di Liberto, B., Pizzo, I., and Campisi, G.**, *Effects of two essential oil mouthrinses on 4-day supragingival plaque regrowth: a randomized cross-over study.* Am J Dent, 2013. 26(3): p. 156-60.

37. **Ulkur, F., Arun, T., and Ozdemir, F.**, *The effects of three different mouth rinses in a 4-day supragingival plaque regrowth study.* Eur J Dent, 2013. 7(3): p. 352-8.

38. **Marchetti, E., Casalena, F., Capestro, A., Tecco, S., Mattei, A., and Marzo, G.**, *Efficacy of two mouthwashes on 3-day supragingival plaque regrowth: a randomized crossover clinical trial.* Int J Dent Hyg, 2017. 15(1): p. 73-80.

39. **Marchetti, E., Tecco, S., Caterini, E., Casalena, F., Quinzi, V., Mattei, A., and Marzo, G.**, *Alcohol-free essential oils containing mouthrinse efficacy on three-day supragingival plaque regrowth: a randomized crossover clinical trial.* Trials, 2017. 18(1): p. 154.

40. **Faul, F., Erdfelder, E., Lang, A.G., and Buchner, A.**, *G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences.* Behav Res Methods, 2007. 39(2): p. 175-91.

41. **Prada-López, I., Quintas, V., Casares-De-Cal, M.A., Suárez-Quintanilla, J.A., Suárez-Quintanilla, D., and Tomás, I.**, *Ex vivo vs. in vivo antibacterial activity of two antiseptics on oral biofilm.* Front Microbiol, 2015. 6: p. 655.

42. **Prada-López, I., Quintas, V., Donos, N., Suárez-Quintanilla, D., and Tomás, I.**, *Characteristics of in situ oral biofilm after 2 and 4 days of evolution.* Quintessence Int, 2015. 46(4): p. 287-298.

43. **World Health Organization**, *Oral health surveys: basic methods*. 5th ed. 2013, France. 125.

44. **Dallal, G.E.,** Available from: www.randomization.com. Last access on 03/09/2017.

45. **Core Team.** *R: A language and environment for statistical computing.* 2016; Available from: http://www.R-project.org. Last access on 14/09/2017.

46. **Netuschil, L., Reich, E., Unteregger, G., Sculean, A., and Brecx, M.**, *A pilot study of confocal laser scanning microscopy for the assessment of undisturbed dental plaque vitality and topography.* Arch Oral Biol, 1998. 43(4): p. 277-85.

47. **Zaura-Arite, E., van Marle, J., and ten Cate, J.M.**, *Conofocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm.* J Dent Res, 2001. 80(5): p. 1436-40.

48. **Al-Ahmad, A., Wunder, A., Auschill, T.M., Follo, M., Braun, G., Hellwig, E., and Arweiler, N.B.**, *The in vivo dynamics of Streptococcus spp., Actinomyces naeslundii, Fusobacterium nucleatum and Veillonella spp. in dental plaque biofilm as analysed by five-colour multiplex fluorescence in situ hybridization.* J Med Microbiol, 2007. 56(Pt 5): p. 681-7.

49. **Auschill, T.M., Hellwig, E., Sculean, A., Hein, N., and Arweiler, N.B.**, *Impact of the intraoral location on the rate of biofilm growth.* Clin Oral Investig, 2004. 8(2): p. 97-101.

50. **Auschill, T.M., Hein, N., Hellwig, E., Follo, M., Sculean, A., and Arweiler, N.B.**, *Effect of two antimicrobial agents on early in situ biofilm formation.* J Clin Periodontol, 2005. 32(2): p. 147-52.

51. **Arweiler, N.B., Hellwig, E., Sculean, A., Hein, N., and Auschill, T.M.**, *Individual vitality pattern of in situ dental biofilms at different locations in the oral cavity.* Caries Res, 2004. 38(5): p. 442-7.

52. **Pizzo, G., La Cara, M., Licata, M.E., Pizzo, I., and D'Angelo, M.**, *The effects of an essential oil and an amine fluoride/stannous fluoride mouthrinse on supragingival plaque regrowth.* J Periodontol, 2008. 79(7): p. 1177-83.

53. **Rosin, M., Welk, A., Kocher, T., Majic-Todt, A., Kramer, A., and Pitten, F.A.**, *The effect of a polyhexamethylene biguanide mouthrinse compared to an essential oil rinse and a chlorhexidine rinse on bacterial counts and 4-day plaque regrowth.* J Clin Periodontol, 2002. 29(5): p. 392-9.

54. **Moran, J., Addy, M., and Newcombe, R.**, *A 4-day plaque regrowth study comparing an essential oil mouthrinse with a triclosan mouthrinse.* J Clin Periodontol, 1997. 24(9 Pt 1): p. 636-9.

55. **Jenkins, S., Addy, M., and Newcome, R.**, *Triclosan and sodium lauryl sulphate mouthrinses. (II). Effects of 4-day plaque regrowth.* J Clin Periodontol, 1991. 18(2): p. 145-8.

56. **Addy, M., Jenkins, S., and Newcombe, R.**, *The effect of triclosan, stannous fluoride and chlorhexidine products on: (I) Plaque regrowth over a 4-day period.* J Clin Periodontol, 1990. 17(10): p. 693-7.

57. **Arweiler, N.B., Lenz, R., Sculean, A., Al-Ahmad, A., Hellwig, E., and Auschill, T.M.**, *Effect of food preservatives on in situ biofilm formation.* Clin Oral Investig, 2008. 12(3): p. 203-8.

58. **Auschill, T.M., Arweiler, N.B., Netuschil, L., Brecx, M., Reich, E., and Sculean, A.**, *Spatial distribution of vital and dead microorganisms in dental biofilms.* Arch Oral Biol, 2001. 46(5): p. 471-6.

59. **Jentsch, H., Hombach, A., Beetke, E., and Jonas, L.**, *Quantitative transmission electron microscopic study of dental plaque--an in vivo study with different mouthrinses.* Ultrastruct Pathol, 2002. 26(5): p. 309-13.

60. **Jentsch, H., Mozaffari, E., and Jonas, L.**, *Scanning electron microscopy of growing dental plaque: a quantitative study with different mouth rinses.* Ultrastruct Pathol, 2013. 37(4): p. 233-40.

61. **Al-Ahmad, A., Wiedmann-Al-Ahmad, M., Auschill, T.M., Follo, M., Braun, G., Hellwig, E., and Arweiler, N.B.**, *Effects of commonly used food preservatives on biofilm formation of Streptococcus mutans in vitro.* Arch Oral Biol, 2008. 53(8): p. 765-72.

62. **Pratten, J., Barnett, P., and Wilson, M.**, *Composition and susceptibility to chlorhexidine of multispecies biofilms of oral bacteria.* Appl Environ Microbiol, 1998. 64(9): p. 3515-9.

63. **Wood, S.R., Kirkham, J., Shore, R.C., Brookes, S.J., and Robinson, C.**, *Changes in the structure and density of oral plaque biofilms with increasing plaque age.* FEMS Microbiol Ecol, 2002. 39(3): p. 239-44.

64. **Herrera, D., Roldán, S., Santacruz, I., Santos, S., Masdevall, M., and Sanz, M.**, *Differences in antimicrobial activity of four commercial 0.12% chlorhexidine mouthrinse formulations: an in vitro contact test and salivary bacterial counts study.* J Clin Periodontol, 2003. 30(4): p. 307-14.

65. **Vlachojannis, C., Chrubasik-Hausmann, S., Hellwig, E., and Al-Ahmad, A.**, *A Preliminary Investigation on the Antimicrobial Activity of Listerine(R), Its Components, and of Mixtures Thereof.* Phytother Res, 2015. 29(10): p. 1590-4.

66. **Petersen, F.C., Assev, S., and Scheie, A.A.**, *Combined effects of NaF and SLS on acid- and polysaccharide-formation of biofilm and planktonic cells.* Arch Oral Biol, 2006. 51(8): p. 665-71.

67. **Ledder, R.G., Sreenivasan, P.K., DeVizio, W., and McBain, A.J.**, *Evaluation of the specificity and effectiveness of selected oral hygiene actives in salivary biofilm microcosms.* J Med Microbiol, 2010. 59(Pt 12): p. 1462-8.

68. **Waaler, S.M., Rolla, G., Skjorland, K.K., and Ogaard, B.**, *Effects of oral rinsing with triclosan and sodium lauryl sulfate on dental plaque formation: a pilot study.* Scand J Dent Res, 1993. 101(4): p. 192-5.

69. **Robinson, C., Strafford, S., Rees, G., Brookes, S.J., Kirkham, J., Shore, R.C., Watson, P.S., and Wood, S.**, *Plaque biofilms: the effect of chemical environment on natural human plaque biofilm architecture.* Arch Oral Biol, 2006. 51(11): p. 1006-14.

70. **Anwar, H., Strap, J.L., and Costerton, J.W.**, *Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy.* Antimicrob Agents Chemother, 1992. 36(7): p. 1347-51.

71. **Boutibonnes, P., Gillot, B., Auffray, Y., and Thammavongs, B.**, *Heat shock induces thermotolerance and inhibition of lysis in a lysogenic strain of Lactococcus lactis.* Int J Food Microbiol, 1991. 14(1): p. 1-9.

72. **Piper, P.W., Talreja, K., Panaretou, B., Moradas-Ferreira, P., Byrne, K., Praekelt, U.M., Meacock, P., Recnacq, M., and Boucherie, H.**, *Induction of major heat-shock proteins of Saccharomyces cerevisiae, including plasma membrane Hsp30, by ethanol levels above a critical threshold.* Microbiology, 1994. 140 (Pt 11): p. 3031-8.

73. **Gjermo, P., Baastad, K.L., and Rolla, G.**, *The plaque-inhibiting capacity of 11 antibacterial compounds.* J Periodontal Res, 1970. 5(2): p. 102-9.

74. **Gordon, J.M., Lamster, I.B., and Seiger, M.C.**, *Efficacy of Listerine antiseptic in inhibiting the development of plaque and gingivitis.* J Clin Periodontol, 1985. 12(8): p. 697-704.

75. **Zamora-Pérez, A.L., Mariaud-Schmidt, R.P., Fuentes-Lerma, M.G., Guerrero-Velazquez, C., Gomez-Meda, B.C., Lopez-Verdin, S., and Zuniga-Gonzalez, G.M.**, *Increased number of micronuclei and nuclear anomalies in buccal mucosa cells from people exposed to alcohol-containing mouthwash.* Drug Chem Toxicol, 2013. 36(2): p. 255-60.

76. **Iacopino, A.**, *Use of alcohol-containing rinses to reduce oral microbial burden: safety and efficacy.* JCDA, 2009. 75: p. 260-261.

77. **Eliot, M.N., Michaud, D.S., Langevin, S.M., McClean, M.D., and Kelsey, K.T.**, *Periodontal disease and mouthwash use are risk factors for head and neck squamous cell carcinoma.* Cancer Causes Control, 2013. 24(7): p. 1315-22.

78. **Garro, A.J. and Lieber, C.S.**, *Alcohol and cancer.* Annu Rev Pharmacol Toxicol, 1990. 30: p. 219-49.

CONCLUSIONS

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1. A single mouthwash of the traditional formulation of essential oils presented very high immediate antibacterial effect *in situ* and a substantivity which lasted for at least 7 hours after its application over *de novo* undisturbed plaque-like biofilm. These results were even better than those observed with 0.2% chlorhexidine under the same conditions.

   Consequently, a single mouthwash of essential oils containing alcohol is an effective measure against the *de novo* oral biofilm, representing a good alternative to chlorhexidine such as a preoperative rinse, in periodontal procedures or post-treatment applications.

2. In a 4-day *in situ* undisturbed plaque-like biofilm model, the traditional formula based on essential oils showed a very high antiplaque effect. Essential oils had a very high antibacterial activity and similar to that detected with the 0.2% chlorhexidine, although the latter presented better results at reducing the thickness and covering grade by the plaque-like biofilm.

   Consequently, the traditional essential oils solution is a reliable alternative to chlorhexidine to prevent its side effects when used continuously.

3. In a 2-day *in situ* undisturbed plaque-like biofilm model, after a single mouthwash, both essential oil formulations had very high immediate antibacterial activity and a substantivity which lasted for at least 7 hours after application. In a 4-day *in situ* undisturbed plaque-like biofilm model, both essential oil formulations demonstrated a very

good antiplaque effect, although the alcohol-free formula performed better at reducing the biofilm thickness and covering grade.

Consequently, the alcohol-free essential oils solution represents a reliable option as antibacterial and antiplaque agent for the control of oral biofilm.